

**Triploid Induction in the South African Abalone, *Haliotis***  
***midae***

**Thesis**

Submitted in fulfilment of a Master of Science Degree in Zoology

University of Cape Town

**Nigel Keith Stepto**

Submitted January 1997

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

**ABSTRACT:**

An investigation was undertaken to determine whether triploidy could be induced and improve the growth rate of the South African abalone, *Haliotis midae*. From the polar body counts at 17 °C the release times of polar body 1 and 2 were found to be at 12 - 15 min. post insemination (pi.) and 33 - 36 min. pi. respectively. Cytochalasin B (CB) (0.5mg. l<sup>-1</sup> seawater) and elevated temperature (30 °C) were used as stresses to induce triploidy. CB induced 48.4% polar body 1 and 70.9 % polar body 2 triploid larvae at 20 hrs pi. At 120 hrs pi. induction rates were 55.5% and 62.4% respectively. Temperature induction was more successful, producing 92.9% polar body 1 and 86.4% polar body 2 triploid larvae at 20 hrs pi. This success was still evident at 120 hrs pi, where 71.1% polar body 1 and 62.5% polar body 2 triploid larvae were produced.

In the CB induction, where polar body 1 was retained, there was a pronounced production of tetraploid larvae (34. 2%). It appeared that CB affected the ova's resistance to polyspermy, which was found to be dependent on both CB concentration and the amount of excess sperm present. At 0.4 mg l<sup>-1</sup> CB in seawater, 86.5% triploids and 0% tetraploids were produced. However, on the addition of sperm, 25.4% triploids and 69% tetraploids resulted. CB (0.5 mg l<sup>-1</sup>) in seawater produced 42.6% tetraploids which, after the addition of sperm, increased even further to 50.1% pentaploids.

Larval survival was found to be low overall with only 17% and 22% of control (diploid) animals surviving the rearing period, in the temperature

and CB treatments respectively. The survival rates of the polar body 2 treatment were 11% and 15% whilst those of the polar body 1 treatment were 7% and 11%. Although these percentages indicated a difference in survival rates between the CB and temperature inductions, the actual numbers of larvae surviving were the same. The polar body 1 triploid larval survival was significantly less than both control and polar body 2 triploid animals.

Although significant differences were difficult to document, there appeared to be a trend for triploid *H. midae* to grow faster than the diploid animals by 0.01 -0.02 mm day<sup>-1</sup>. When trying to decide whether polar body 1 or 2 triploid abalone were better, no definite conclusions could be drawn, even though the polar body 1 triploids had slightly faster growth rates. Thus the perceived growth improvement in triploid *H. midae* will require further investigation to validate the results.

**ACKNOWLEDGEMENTS:**

In completing this research I had to rely on a number of people for various aspects of the research and I would like to extend my gratitude and thanks for their help and patience. Firstly my supervisor Associate Professor Cook for supporting me with both funding and knowledge. Irvin & Johnson Pty. (Ltd) for providing the funding and facilities to do the research, the farm manager and staff for all their help and advice, and especially Mike Boon for the great hospitality he offered when I stayed on the farm. I would also like to thank the many patient people and technicians who helped out with that meanest of temperamental beasts, the Flowcytometer, Dr Pauline Lukey, Dr John Michie, Dr Trevor Probyn and the Coulter Electronics Technicians. The UCT Zoology departments technicians Bruce Dell for the wonders he performed in the workshop. Finally, my family and especially my father for all the proof reading and corrections.

**CHAPTER 1:**

**Ploidy Manipulations in Shellfish**

University of Cape Town

Aquaculture in South Africa has been in existence since 1890 (Hecht & Britz 1990) when the initial fresh water fish farms were established. More recently, as the freshwater fish industry has reached saturation, (Hecht 1994) the attention of aquaculture has shifted to the marine environment, where shellfish such as oysters and mussels are now considered to be good culture animals (Hecht & Britz 1990). The South African abalone, *Haliotis midae*, also shows promise as a culture organism. Abalone culture is now on the verge of commercial production in South Africa, and appears to be a very promising enterprise. One of the reasons for this is that the global abalone fisheries, including the small local fishery, are experiencing yearly yield declines (Britz 1990). This has yet to decrease the demand for this prized commodity in the Far East (Featherstone 1993).

South African farms will probably produce the small 'cocktail' abalone (about 80 mm shell length), but *H. midae* is a very slow growing species (Newman 1968). Much of the research to date has focused on creating optimal conditions and diets for these animals (Genade, Hirst & Smit 1988; Simpson 1992; 1995), but room for improvements in growth rates still exists. Use of genetic manipulations to improve growth rates had already shown

promise for other species of abalone (Fallu 1991; Fujino 1992) and other valuable culture molluscs (Purdom 1983; Beaumont & Fairbrother 1991; Thorgaard, Galbreath & Young 1995).

Genetic manipulations of both fish and shellfish have been studied for about the past 10 years. Initially, these manipulations were researched for commercially valuable salmonoid fish species (Purdom 1983; Thorgaard 1983; Chourrout 1987). These types of manipulations usually involve alteration of the maternal genome. Ploidy manipulations are often combined with intensive selective breeding, further improving the net effect of the genetic manipulations (Purdom 1983; Fujino 1992). Fish and shellfish genome manipulations are similar, both providing improved growth rates by sterilizing the animals to uncouple the energy used for reproduction (Davis 1986,1989; Mason, Shumway, Allen & Hidu 1988; Allen & Downing 1991) and increasing heterozygosity (Stanley, Hidu & Allen 1984; Allen 1987; Allen 1988; Hawkins, Day, Gerard, Naciri, Ledu, Bayne & Heral 1994). In many cases, such manipulations have proved successful and are now under intensive investigation for shellfish farming (Beaumont & Fairbrother 1991) including abalone (Arai, Naito & Fujino 1986; Fujino 1992; Fallu 1991).



Genetic enhancements can be placed into three categories of ploidy manipulations: triploidy, gynogenesis and tetraploidy. Each of these will be discussed further with respect to shellfish but triploidy will be the main focus of discussion as the other two are mainly for comparison to show the subtle differences in the techniques used to manipulate the chromosomes and final genetic make up. The resultant ploidy may differ in each of the three types of manipulations. Improved growth resulting from ploidy manipulations has been shown in oysters (Allen & Downing 1990; Mann, Burrenson & Allen 1994), abalone (Arai *et al.* 1986; Fujino, Arai, Iwadare, Yoshida & Nakajima 1990), clams (Allen, Gagnon & Hidu 1982; Beaumont & Contaris 1988; Dufy & Diter 1990), mussels (Beaumont & Kelly 1989; Toro & Sastre 1995) and scallops (Tabarini 1984; Baron, Diter & Bodoy 1989). When tetraploids are produced the problem appears to be survival. A few tetraploid oysters, (Stephens & Downing 1989) and clams (Diter & Dufy 1990; Allen, Sphigel, Utting & Spencer 1994) have survived, but so far the value of tetraploids has been difficult to assess (Allen *et al.* 1994).

Ploidy manipulations are possible because of the specialized mechanism of maturation of the molluscan egg (Longo 1983; Komaru, Matsuda, Yamakawa & Wada 1990). During the process of sexual reproduction, the germ cells undergo two maturation divisions before becoming active gametes (Fig. 1.1). In the first meiotic division (meiosis 1) homologous chromosomes associate and form a tetrad of sister chromatids. Recombination of genetic material results from the crossover between non-sister chromatids. The genetic material is halved at the end of meiosis 1 as one chromosome from each homologous pair is taken into each of the daughter cells. Meiosis 2, the second maturation division, follows immediately after and is similar to mitosis, where each of the four daughter cells receives one chromatid. Sexual reproduction is then completed when syngamy (the uniting of a female and a male gamete) has occurred (Longo 1983; Komaru *et al.* 1990).

Male germ cells produce four sperm cells, and the female germ cells produce a single ovum, once meiosis 1 and 2 are completed. The excess genetic material resulting from meiosis 1 and 2, in the female cells, are removed in the form of polar bodies 1 and 2 respectively. Polar bodies are small extrusions containing the genetic material and very little cytoplasm. The

suppression of release of these extrusive bodies allows for genome manipulations. This process can occur in nature and has been termed parthenogenesis. Generally some type of environmental cue causes the retention of one of the polar bodies in an unfertilized egg, resulting in the retention of the diploid state of the zygote. The artificial induction of polar body retention has resulted in triploid, gynogenetic diploid and tetraploid shellfish (Beaumont & Fairbrother 1991). Abalone, have a similar egg maturation (Chapter 2) it is thus possible to perform ploidy manipulation on this genus which Arai *et al.* (1986) have shown to improve growth in *H. discus hannai*. These results suggest the possible value of triploidy research to the farming of abalone.

Triploidy results when a cell nucleus contains three sets of chromosomes, the extra set of chromosomes preventing meiosis from occurring (Longo 1972, 1983; Purdom 1983). Triploid animals are sterile which means that energy that would normally be used for reproductive purposes can be diverted to metabolic functions (Davis 1986, 1989; Allen 1988; Hawkins *et al.* 1994; Laing & Utting 1994). Other genetic

considerations (such as increased heterozygosity) should, however, not be ignored when considering the effect of triploidy.

Increased heterozygosity is important because it is reported to result in faster growth (Fujio 1982; Zouros & Pogson 1994). This has also been demonstrated in animals such as abalone (Arai *et al.* 1986; Fujino 1987, 1992), clams (Beaumont & Contaris 1988; Nell, O'Connor, Hand & MacAdam 1995), oysters (Stanley, Allen & Hidu 1981; Scarpa, Vaughan & Longley 1995), mussels (Yamamoto & Sugawara 1988; Scarpa, Toro & Wada 1994) and scallops (Tabarini 1984; Toro, Sanhueza, Paredes & Canello 1995), where the more heterozygous triploids have shown better growth. The reason for this stems from the two types of triploidy that can be induced. The first type is PB1 triploidy which results from the retention of the first polar body, where all the maternal DNA is retained. The second type is PB2 (polar body two) triploidy where the second polar body is retained and only half the maternal DNA is kept (less heterozygous), and the animals' corresponding growth rates are similar to the normal diploid growth animals (Allen & Downing 1986; Fujino 1992). The two types of triploidy can be distinguished

by the timing of the induction process (release of the polar bodies) which is usually unique to the species concerned.

The effects of triploidy have been examined in many molluscan species including abalone, clams, mussels, oysters and scallops (Table 1.1). In most cases, both types of triploidy could be induced but in general it appears that PB1 triploids produced better growth rates.

A number of methods have been employed to induce triploidy in the molluscs including chemical induction (Cytochalasin B and 6-DMAP), pressure stress, thermal stress and the application of an electric field shock (Table 1.1).

TABLE 1.1: Summary of the triploid inductions performed on shellfish, where % 3N is the amount of triploidy induced, CB = induction with cytochalasin B (if no concentration mentioned then it was 0.05 mg l<sup>-1</sup>), 6-DMAP = induction with 6-DMAP, Press = induction with pressure, Temp = temperature induction.

Species	Author	Method of induction	Best % 3N	Date
<b>ABALONE</b>				
<i>Haliotis discus hannai</i>	Arai et al.	Temp (3°C)	80	1986
		(35°C)	80	
		Press (200 kg.cm <sup>-2</sup> )	60	
<b>CLAMS</b>				
<i>Mya arenaria</i>	Allen et al.	CB	100	1982

Species	Author	Method of induction	Best % 3N	Date
<i>M. arenaria</i>	Mason et al.	CB	44	1988
<i>Tapes semidecussatus</i>	Beaumont & Contaris	CB	68	1988
<i>T. semidecussatus</i>	Gosling & Nolan	Temp (32°C)	55	1989
		CB	50	
<i>Ruditapes philippinarum</i>	Dufey & Diter	CB (1 mg l <sup>-1</sup> )	76	1990
<i>R. philippinarum</i>	Utting & Child	CB	77	1994
<i>T. dorsatus</i>	Nell et al.	CB (1 mg l <sup>-1</sup> )	85	1995
<b>MUSSELS</b>				
<i>Mytilus edulis</i>	Yamamoto & Sugawara	Temp (1°C)	85	1988
		(32°C)	97	
<i>M. edulis</i>	Beaumont & Kelly	Temp (32°C)	25	1989
<i>M. galloprovincialis</i>	Scarpa et al.	CB (1 mg l <sup>-1</sup> )	81	1994
		Temp (30°C)	86	
<i>M. chilensis</i>	Toro & Sastre	Temp (32°C)	51	1995
<b>OYSTERS</b>				
<i>Crassostrea gigas</i>	Allen, Downing, Chaiton & Beattie	CB (1 mg l <sup>-1</sup> )	100	1986
		Press (7200 psi)	60	
<i>C. gigas</i>	Quillet & Panelay	Temp (35°C)	76	1986
		(32°C)	61	
<i>C. gigas</i>	Downing & Allen	CB (1 mg l <sup>-1</sup> )	88	1987
<i>C. gigas</i>	Yamamoto, Sugawara, Normura & Oshino	CB	67	1988
		Temp (37°C)	83	
<i>C. gigas</i>	Scarpa et al.	6-DMAP	-	1995
<i>C. virginica</i>	Stanley et al	CB (0.1 mg l <sup>-1</sup> )	59	1981
		CB	50	
		CB (1 mg l <sup>-1</sup> )	75	
<i>C. virginica</i>	Allen & Bushek	CB	79	1992
<i>C. virginica</i>	Barber, Mann & Allen	CB (0.25 mg l <sup>-1</sup> )	98	1992
		CB	99	
<i>Ostrea edulis</i>	Grendeau & Grizel	CB (1 mg l <sup>-1</sup> )	70	1990
<i>Pinctada fucata</i>	Wada, Komura &	CB	100	1989
<i>martensii</i>	Uchimura	CB (0.1 mg l <sup>-1</sup> )	80	
		Temp (6.5°C)	52	
<i>P. martensii</i>	Shen, Zhang, He & Ma	Press (250 kg cm <sup>-2</sup> )	76	1993
<b>SCALLOPS</b>				
<i>Argopecten irradians</i>	Tarabini	CB	94	1984
<i>A. purpuratus</i>	Toro et al	Temp (31°C)	66	1995
<i>Chlamys nobilis</i>	Komura, Uchimura, Ieyama & Wada	CB	88	1988

Species	Author	Method of induction	Best % 3N	Date
<i>C. nobilis</i>	Komura & Wada	CB	71	1989
<i>C. varia</i>	Baron et al.	CB	78	1989
<i>Pecten maximus</i>	Beaumont	CB	30	1986

The first, and most common, method is the use of a fungal metabolite (*Helminthosporium dematioideum*) known as Cytochalasin B (CB), which disrupts the cleavage of cells by inhibiting the polymerization of the actin filaments. It thereby prevents the segregation and removal of genetic material from the maturing egg, but does not affect the other cellular processes of cell division (Krishan 1972; Longo 1972, 1983; Copeland 1974; Peaucellier, Guerrier & Bergerard 1974; Schroeder 1978). CB is used at concentrations ranging from 0.1 to 1 mg l<sup>-1</sup>, at temperatures of 15°C to 25°C and the treatments generally last for 15 to 20 minutes. The actual treatment depends on the species involved (Downing & Allen 1987). As CB is a hydrophobic substance it must be dissolved in DMSO (dimethyl sulphoxide) a carrier solution (Allen 1987; Allen, Downing & Chew 1989) prior to addition to sea water. The timing of the treatments of the fertilized eggs corresponds with the initial release of the first or second polar body and lasts 15 - 30 minutes. After treatment, embryos are rinsed in a 0.01% DMSO and filtered seawater for 15

- 20 minutes (Allen *et al.* 1989). Thereafter the eggs are returned to seawater and reared normally. The use of CB seems to be a simple method and appears to give some of the best results (up to 100% triploids (Table 1.1)) in terms of triploid induction.

More recent studies on induction methods have been achieving similar success with the chemical 6-DMAP (Scarpa *et al.* 1995). Its advantages are that it has the same action as CB but is water soluble and far less toxic.

The second method, pressure shock, has successfully produced triploid oysters (Allen & Downing 1986; Chaiton & Allen 1985) and abalone (Arai *et al.* 1986; Fujino 1987, 1992)(Table 1.1). Fertilized eggs were pressurized to 200 - 250 kg.cm<sup>-2</sup> (Chaiton & Allen 1985; Fujino 1987) or 6000 - 8000 psi (Allen *et al.* 1986) for 10 min. over the period when the polar bodies were being released (determined prior to treatments). The high pressure interferes with normal meiotic and mitotic cell function and must therefore be reversed after the treatment period to continue development under normal conditions. The success rates of triploid induction by pressure have been up to 76% , which indicates that pressure may be the least effective method (Table 1.1).



The third method is thermal shock, where both hot and cold treatments have been used. Temperatures commonly used for heat shocks are between 25 - 38 °C and cold shocks between 0 - 7°C (Table 1.1). The temperature differential between the incubation and treatment temperatures, is of more importance than the absolute temperature (Allen 1987; Beaumont & Fairbrother 1991). Thermal shock treatments have been successfully carried out on abalone, clams, mussels, oysters and scallops with induction rates of from 50 - 80% (Table 1.1).

Temperature and chemical shocks have been combined for more effective induction methods (Downing & Allen 1987; Scarpa, Toro & Wada 1994).

The final method, electric field shock, is an extremely specialized and complex process and probably would not be commercially viable as it would be very labour intensive. Previous studies showed that mussel zygotes required specific orientations of the fertilized eggs (Cadoret

1992). An electric field of  $600 \text{ V cm}^{-1}$  for 50 - 200 ms at 1 - 6 pulses was applied (Cadoret 1992). The problem with the method was that the zygotes had to be individually manipulated, to place them in the correct orientation in the electric field, for the treatment to be successful. Large scale production was not a success (Cadoret 1992) and would thus be very time consuming and not a viable induction method for abalone farming.

Triploidy has resulted in increased growth rates, mainly from polar body 1 induction. Larval mortality was high in all cases where triploidy was induced, but in mariculture this seems not to be much of a problem as often many millions of eggs can be produced (Beaumont & Fairbrother 1991; Fallu 1991; Fujino 1992). The implication of triploidy in mariculture, if the methods can be reproduced at a commercial level, is that farmed abalone, or other shellfish, will have much reduced rearing times and thus reduced production costs.

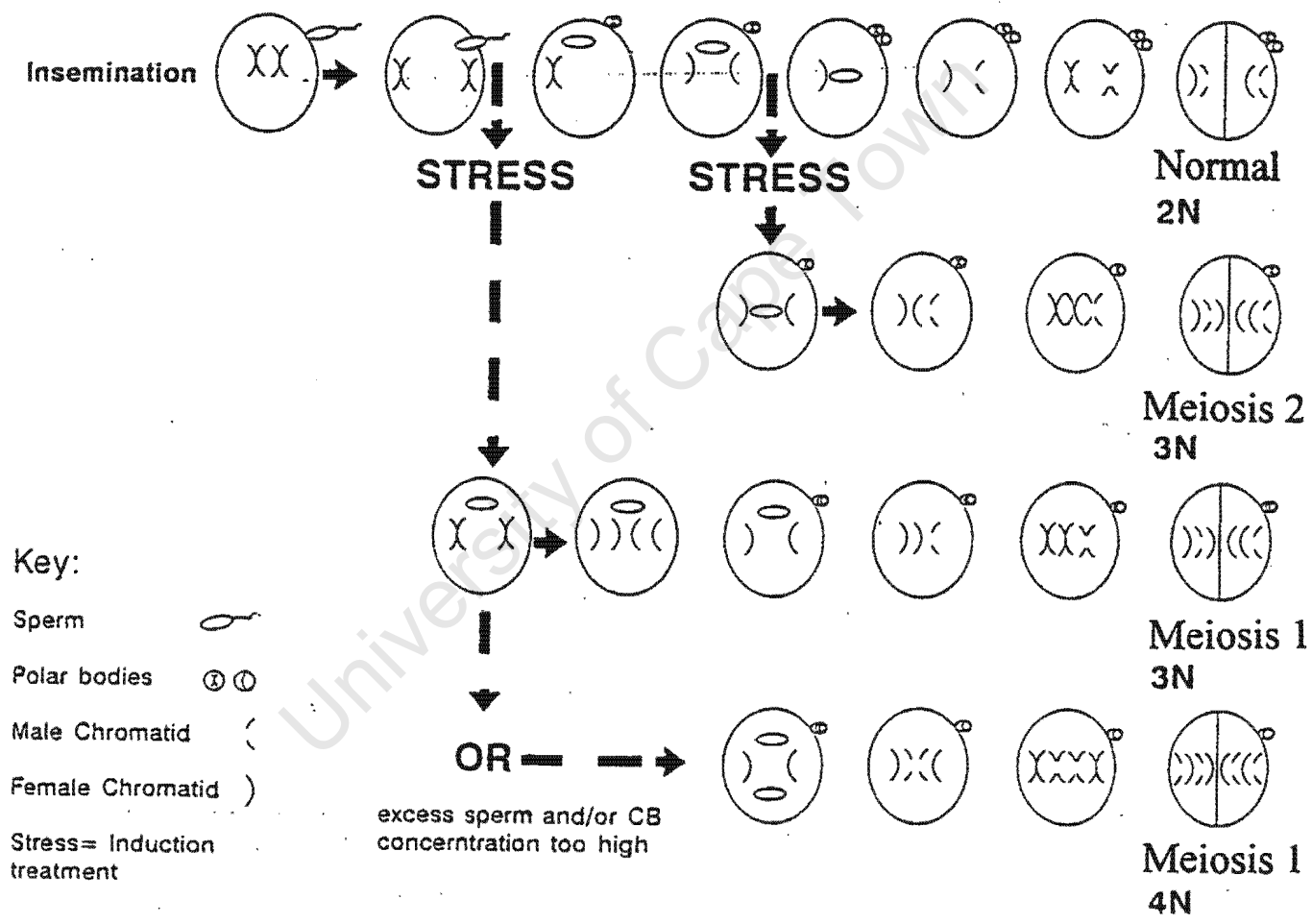


Figure 1.1: Diagrammatic representations of events occurring during fertilization and when the polar bodies are retained to produce triploids or even polyploid shellfish.

Once the ploidy manipulations have been carried out, one needs to assess the success of the manipulations as soon as possible to avoid unnecessary space and time wastage on rearing larvae with low yields of polyploids. Allen (1987) and Beaumont & Fairbrother (1991) have listed techniques that can be used to measure the ploidy. Methods include chromosome counts, polar body counts, flowcytometry, microfluorometry, nuclear sizing by microscope or Coulter counting and electrophoresis. Each of these will be discussed in conjunction with suitability to rapid ploidy detection.

1) Chromosome counts provide one of the most direct ploidy verifications, and can be used on eggs or adult tissue. The technique is, however, time consuming and it is not uncommon to find that the majority of embryos examined do not produce countable chromosome spreads (Allen 1987; Beaumont & Fairbrother 1991). It still remains the most direct and least expensive method for ploidy verification.

2) Polar body counting in young embryos has been suggested as a rapid and simple method of assessing the effectiveness of triploidy in bivalves (Beaumont & Contaris 1988; Beaumont & Kelly 1989). As triploid induction involves the retention of a polar body, a successful triploid embryo should possess only one polar body. This method, however, is only useful if the polar bodies can be easily distinguished. It may not be suitable for abalone as *H. midae* polar bodies are not distinct (personal observations). Stephens and Downing (1988) have gone a step further using the DAPI stain (4'-6-diamino-2-phenylindole) to determine the ploidy of the polar bodies. Since PB1 is diploid and PB2 is haploid the method can clarify which maturation division was effected by the shock treatment.

3) Flow cytometry involves obtaining a single cell suspension and staining the nuclei with an epifluorescent, DNA-RNA specific dye, either propidium iodide or DAPI. The stained cells are passed through a cytographic analyser in a liquid suspension, where the intensity of fluorescence is recorded (Allen 1983). Mantle tissue, haemolymph, frozen tissue (Allen 1983), single larvae (Chaiton & Allen 1985) and one day old larvae (Downing 1989) have been used to provide cells for assay purposes. Samples are analysed with an

internal standards, such as chicken erythrocytes or the animal's own sperm (Allen 1983; Vindelov, Christensen & Nissen 1983), to determine the relative DNA contents. Modal pulse height histograms are recorded, where the triploid modal values should be 1.5 times greater than the diploid values. Flow cytometry is a fast and accurate procedure, which does not always necessitate the sacrificing of adult animals (Allen 1983). In the case of abalone *H. midae*, however, animals generally have to be sacrificed because of an inability to heal wounds (Cox 1962; Michelson 1975; Taylor, Schiel & Taylor 1994). However, the drawback of this technique is the expense of the flow cytometer and test methods.

4) Microfluorimetry uses the DAPI stain, which is an aromatic fluorochrome which binds preferentially to the adenine-thiamine base pairs of DNA (Hamada & Fujita 1983; Uchimura, Komura, Wada, Ieyama, Yamaki & Furata 1989). The stained nuclei are excited with ultra-violet light and the fluorescent intensity measured with a photometer. A histogram of fluorescence intensity (relative DNA content) produced from a sample containing haploid (sperm), diploid (adult tissue) and triploid (tissue from treated animals) cells would show three distinct peaks (Allen 1988; Komura *et*

*al.* 1988; Uchimura *et al.* 1989), which can demonstrate whether or not ploidy manipulation was successful. Again the technique is expensive, due to the equipment requirement.

5) Cell sizing can be accomplished in two ways, using overall cell size or nucleic sizing. The first method determines cell size with a Coulter counter.

The method was tested by Child & Watkins (1994) as it was believed that a triploid cell would be larger as it carried more DNA than a normal diploid cell.

The results were within acceptable limits, but it was found that a flow cytometer was far more accurate (Johnson, Rabinovich & Utter 1984). The second method of sizing required the manual measurement of the nuclear diameter (appropriately stained). The cell nucleus was assumed as 1.5 X the volume reflected in the nuclear diameter. The results were compared to flow cytometric data and provided an almost identical result (Child & Watkins 1994). Thus sizing, especially nuclear sizing, shows great promise for identifying the induction success, particularly in remote areas, such as farms.

6) The final method is electrophoresis, which is not widely used for verification of ploidy. Allen *et al.* (1982) compared its effectiveness with that

of chromosome counts as verification tools of ploidy in *Mya arenaria*, and suggested that electrophoresis only proved useful when high heterozygosity was present.

There are further ploidy manipulations that have been investigated for use in mariculture. It is interesting to make a brief mention of these other techniques to expose the differences that exist in the methods used to induce these other forms of ploidy manipulations. However, improved growth rate is still the main aim of these genetic manipulations.

Gynogenesis is the production of shellfish from maternal chromosomes only (cloning). It requires selective breeding to produce rapid growing or disease resistant females. However the young are more homozygous than the maternal animal, depending on the timing of the shock treatment used to convert the haploid zygote to a diploid one. This has been researched for some commercially important abalone (Fujino *et al.* 1990) and oysters (Guo, Hershberger, Cooper & Chew 1993).



The procedure requires the use of inactivated sperm, either ultra violet light or gamma-irradiated, to induce egg maturation. The resulting zygotes are haploid, which are returned to the normal diploid state using any one of the above mentioned induction methods such as CB or heat shock (Fairbrother 1994; Fujino *et al.* 1990; Guo *et al.* 1993) . One of these shock treatments would be applied at initial PB1, PB2 release or at first cleavage (Beaumont & Fairbrother 1991).

Homozygous diploid gynogens would result from the above methods, which could be detrimental to the resulting stock if the correct characters (e.g. fast growth) have not been selected prior to the use of this technique. The implications for mariculture are that faster growing clones could be produced. However these gynogens lack heterozygosity and are susceptible to changing environments (Zouros & Pogson 1994) especially in raft based operations, which are exposed to a fluctuating environment.

In tetraploids, cells have the chromosome number or DNA content doubled through ploidy manipulations. According to Beaumont & Fairbrother

(1991) there are three methods to induce tetraploidy, suppression of first cleavage, blastomere fusion, and early shock treatment.

Suppression of cleavage is a theoretical concept where suppression of the first cleavage will result in a cell with double the number of chromosomes.

The subsequent mitotic division would then result in tetraploid cells (Beaumont & Fairbrother 1991). To date this technique has not been used.

Blastomere fusion is a new concept where the blastomeres (two cell embryos) are induced to fuse using a 50% polyethylene glycol solution (Guo and Allen 1994 ). This fusion concept has also been used in combination with mitosis 1 inhibition to form tetraploids (Guo, Hershberger, Cooper & Chew 1994). The method produced tetraploidy but the oysters did not survive for very long (Guo *et al.* 1994).

Lastly, early shock treatments, especially at the stage of meiosis 1 seems to produce tetraploids (Stephens & Downing 1989; Grendreau & Grizel 1990; Guo, Hershberger, Cooper & Chew 1992; Allen *et al.* 1994). Beaumont & Fairbrother (1991) believe that the reason for the production of tetraploids is

that the CB treatments delay sperm entering the egg at activation, which result in tetraploids. Contrary to the latter beliefs, personal findings (Chapter 3) indicate that CB may have a more profound effect on membrane resistance to polyspermy, even in these low concentrations (Longo 1972, 1983). This would allow more than one sperm cell to be incorporated into the zygote, producing tetraploid and pentaploid animals (Chapter 3). However, Guo *et al.* (1992) believe that the resulting tetraploids, which resulted from inhibition of meiosis 1 and the resulting eggs of *Crassostrea gigas*, had variable patterns of chromosome segregation once re-incorporated into the cell and preparing for the next cell division. Two out of the four patterns of segregation resulted in high proportion of the animals being firstly tetraploid, aneuploid and then triploid (Guo *et al.* 1992).

Guo *et al.* (1994) and Guo & Allen (1994) found that tetraploid molluscs have a 100% mortality at this time, but that it should be the future direction for genetic/ploidy manipulation to enhance molluscan growth for mariculture. The reason for the high mortality rate of the tetraploid shellfish is believed to be the fact that the eggs do not have a large enough nutrient store to sustain the increased DNA content (Allen *et al.* 1994; Beaumont & Fairbrother 1991;

Utting & Doyou 1992). More recently Allen *et al.* (1994) have found a few incidental tetraploids (oysters) after a triploid induction. These results bring new hope for tetraploid induction.

In conclusion, it appears that ploidy manipulations may be an important method to improve production of shellfish on farms. However, the most relevant and cost effective method must be selected to perform these ploidy manipulations. The methods that have been selected for induction of triploidy in *H. midae* in the present study are the use of the chemical Cytochalasin B (CB) and thermal (30°C) shock. The success of the inductions will be analysed using flow cytometry.

## **CHAPTER 2:**

### **Polar Bodies**

University of Cape Town

**Introduction:**

Reproduction is a process that begins with the formation of haploid sex cells, produced by a specialised cell division process called meiosis. Meiosis in males produces four sperm cells, whilst females only one ovum per sex cell which is formed by a more specialised meiosis (Longo 1983; Beaumont & Fairbrother 1991). The gametes then fuse and the nuclear material intermixes, producing a single diploid zygote, which then develops into a new animal (Campbell 1990).

In molluscs a ripe ovum is often released when meiosis is in a dormant phase (Longo 1983; Allen 1987, 1988; Hahn 1989; McShane 1992) and the egg is only activated after sperm attachment. At this point, the reduction divisions begin and asters are formed, one of which moves half the chromatin to the polar edge of the cell. The other half is kept in the middle of the cell. The chromatin at the polar edge is moved into a small extrusion which is then cleaved off in a similar manner as a cell would cleave in mitosis. This extrusion is known as polar body 1 (PB1). The cell then continues to divide and moves into the second reduction producing polar body 2 (PB2) subjacent to the first. The process is the same as for

PB1. The remaining chromatin now forms the female pronucleus. Concomitantly the fused sperm cell has entered the ovum and broken down into the male pronucleus. These two pronuclei now fuse to form the new diploid nucleus of the zygote prior to the first cleavage (Longo 1983).

The fact that early development of fertilized ova is delayed in the reduction divisions, viz. the production of PB1 and PB2, to after fertilization, is important for ploidy manipulations, as the retention of excess DNA is required. By the application of various types of stresses to newly fertilized ova it is possible to cause the retention of either PB1 or PB2 and therefore alters the ploidy of the zygote (Longo 1972, 1983; Allen 1987; Beaumont & Fairbrother 1991). Thus correct assessment of the timing of the formation of the polar bodies is essential to the success of ploidy manipulations.

### **Methods:**

The broodstock was prepared for spawning, and all food and detritus was removed from the housing tanks. Male and female animals were spawned separately using hydrogen peroxide and sodium thiosulphate (Hahn 1989). The ova were then collected in basins and placed in

seawater maintained at 17 °C. The ova were then inseminated with excess sperm at a known time. Unused sperm were removed after 15 minutes by washing the ova.

Sampling of the fertilized ova for counting purposes was carried out simultaneously. Samples of 1000 to 2000 ova were preserved in 1% formalin in seawater. Samples were taken every 3 minutes for the first 36 minutes post insemination (pi), and then from 40 to 60 minutes pi samples were taken every 5 minutes. This sampling schedule was adequate to cover the release of the 2 polar bodies and, in some instances, the starting of the first cleavage.

One thousand ova per sample were counted under 80x magnification on a dissecting microscope to determine the timing of the release of the polar bodies.

It was assumed that once 45 % of either polar bodies were found , the entire population had released its polar bodies (Arai *et al.* 1986; Komura *et al.* 1990; Fujino 1992;).

### **Results:**



The results obtained from the counts at 17 °C provided a very useful time schedule of the early development of zygotes of *H. midae*. The timing of the release of polar body 1 (PB1) was between 12 and 15 minutes pi, when 45% of PB1 were counted (Fig. 2.1). The second event, the release of polar body 2 (PB2), occurred between 33 and 36 minutes pi (Fig. 2.1) when again the 45 % level was reached.

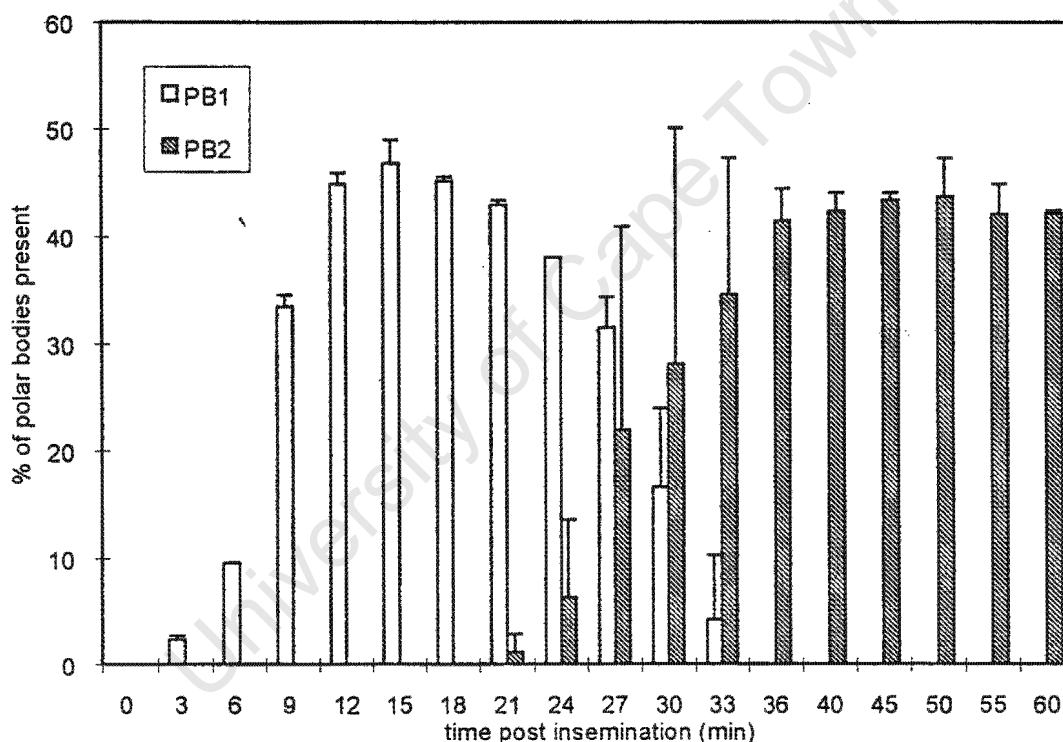


Figure 2.1: Polar body counts (17°C) showing that polar body 1 (PB1) is released at 12 - 15 min., and polar body 2 (PB2) is released at 33 - 36 min..

Figs 2.1 and 2.2 illustrate the timing of the release of the polar bodies and their positioning in relation two each other. PB1 was released at the

animal pole of the ova (Fig. 2.2), then 21 minutes later PB2 was released sub-adjacent to PB1. These protrusions both contain DNA from the reduction divisions of the ova.

### **Discussion:**

In Mollusca, as in other invertebrates, meiosis of the female sex cells into a complete ovum is delayed until insemination (Longo 1972, 1983; Allen 1987). Thus, the development of the polar bodies only occurs after the sperm has inseminated the ovum. This reactivation of the eggs allows the resumption of meiotic maturation (Longo 1983). Part of this process is the reduction divisions requiring the removal of DNA via protrusions called polar bodies.

Polar body formation occurs at the end of anaphase I, where the dyads separate by aster formations (Longo 1983; Komura *et al.* 1990; Beaumont & Fairbrother 1991). One of the asters then moves to a peripheral pole into a cytoplasmic protrusion which then separates from the zygote by a contractile ring (containing microfilaments). At the end of anaphase II the

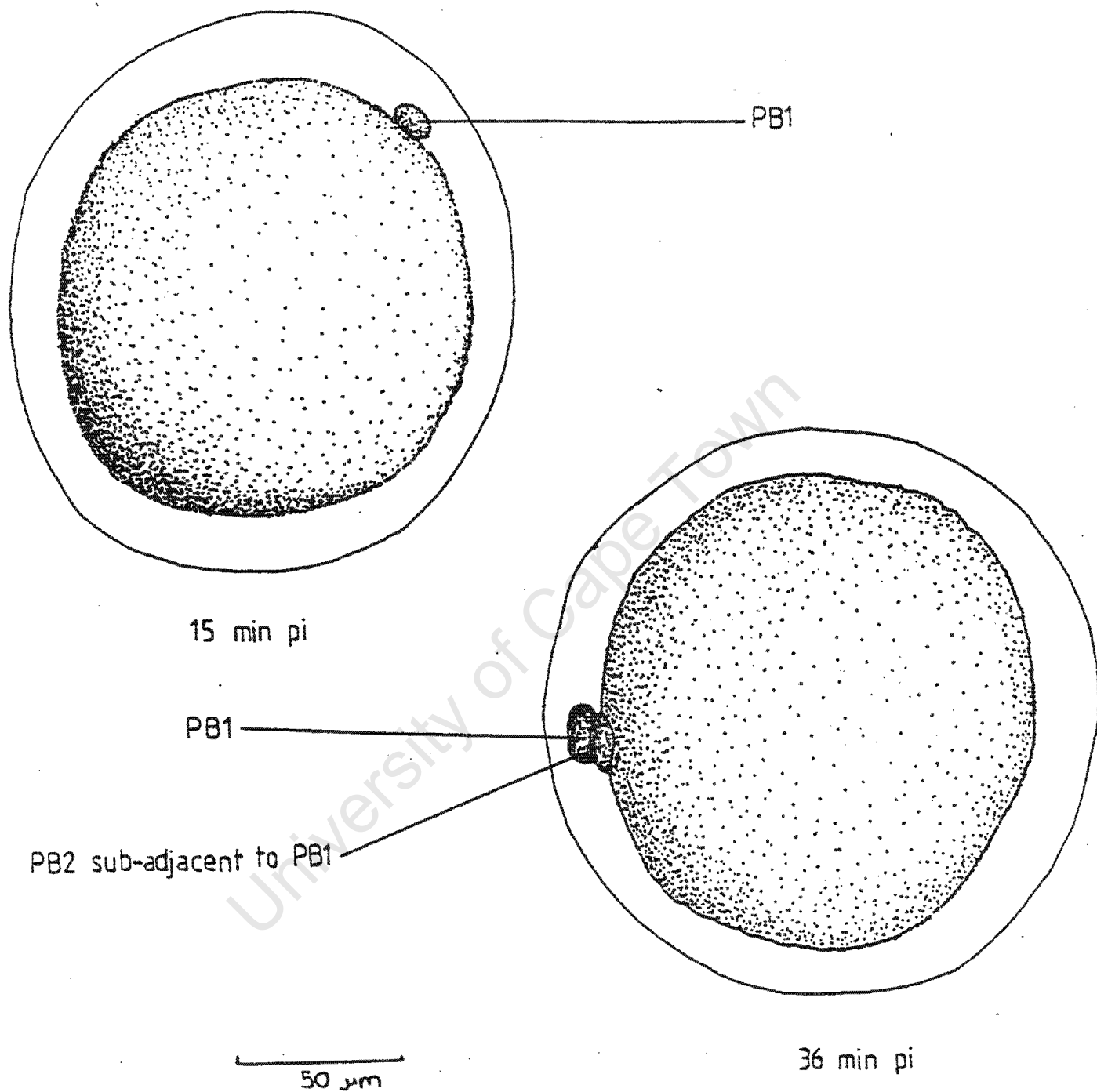


Figure 2.2: Diagram showing the positions of polar bodies 1 (PB1) and 2 (PB2) on the fertilized ova of *Haliotis midiae*. (80 x)

process of separation is repeated, resulting in the release of the second polar body (Longo 1983). This polar body then lies sub-adjacent to the first, as seen here in Fig. 2.2. Similar polar body positioning occurs in *H. discus hannai* (Arai *et al.* 1986) and *Pinctada fucata martensii* (Komura *et al.* 1990).

The removal of genetic material is obviously very important in sexual reproduction, allowing two haploid sex cells (male and female) to form a new diploid zygote. This, however, allows ploidy manipulations if one can prevent either one or both polar bodies from being extruded. Many of the early investigations of the extrusions of polar bodies and the mechanisms of release, used a number of chemicals like concanavalin A, cytochalasin B and lectin, which prevented polar body releases (Longo 1972, 1983). This provided a method of ploidy manipulation which has been used by a number of researchers on many molluscan shellfish (Allen 1987; Beaumont & Fairbrother 1991). Other methods have also been used effectively in preventing polar body release, such as temperature (Arai *et al.* 1986; Gosling & Nolan 1989; Wada Komaru & Uchimura 1989) and pressure (Allen *et al.* 1986; Arai *et al.* 1986; Shen *et al.* 1993) shocks.

Polar body release holds the key to genetic manipulations such as ploidy alteration. Determination of the timing of polar body release allows one to determine when to treat the ova \ zygotes to prevent polar body extrusion. It must also be noted that this timing is temperature dependent (Longo 1983; Arai *et al.* 1986; Hahn 1989; Fujino 1992), the one reported here for *H. midae* being for a water temperature of 17°C. It is, therefore, important during ploidy manipulations, that insemination is carried out at a regulated temperature for which the timing of polar body release has been determined.

**CHAPTER 3:**

**Induction of Triploidy**

University of Cape Town

**Introduction:**

Aquaculturists are now trying to produce large quantities of shellfish, many of which grow relatively slowly in their natural environment. Genetic manipulations could possibly serve as a valuable tool in enhancing these productions (Beaumont & Fairbrother 1991). The induction of triploidy has been identified as one of the most successful forms of genetic manipulation and has been shown to improve growth in other abalone (*H. discus hannai*) (Allen & Downing 1986; Fujino 1992), clams (Buzzi & Manzi 1987; Mason *et al.* 1988; Gosling & Nolan. 1989; Diter & Dufy 1990; Dufy & Diter 1990; Utting & Doyou 1992; Nell *et al.* 1995), mussels (Yamamoto & Sugurawara 1988; Beaumont & Kelly 1989; Toro & Sastre 1995), oysters (Yamamoto *et al.* 1988; Wada *et al.* 1989; Allen & Downing 1990; Allen *et al.* 1989; Barber *et al.* 1992), and scallops (Tabarini 1984; Baron *et al.* 1989; Toro *et al.* 1995). The methods for the induction of triploidy vary from chemical induction (Cytochalsin B (CB)) (Stanley & Allen 1981; Allen *et al.* 1986; Beaumont & Contaris 1988; Cooper & Guo 1989; Utting & Child 1994), pressure stress (250 - 300 kg.cm<sup>-2</sup>) (Allen *et al.* 1986; Beaumont & Contaris 1988; Shen *et al.* 1993) and temperature stress (1 - 5°C or 27 - 30°C) (Arai *et al.* 1986; Quillet & Panelay 1986; Yamamoto & Sugurawara 1988;) which all have a denaturing effect on the newly fertilized egg.

These methods have produced a 60% or higher triploid induction rate (Beaumont & Fairbrother 1991). However, CB has consistently produced 80% and higher induction rates in all the shellfish species tested (Allen 1987; Beaumont & Fairbrother 1991; Fallu 1991). Thus CB was selected as one of the induction agents for *H. midae*.

CB is a fungal metabolite isolated from *Helmenthosporidium dematioiduem*, and was named cytochalasin from the Greek, meaning cell relaxation (Copeland 1974). Early workers noted this reaction when cells were exposed to this chemical (Longo 1972; Copeland 1974; Peaucillier *et al.* 1974; Schroeder 1978). CB was initially used in histological studies, which helped with the discovery of the contractile ring which is used by cells for cell separation after cell division (Copeland 1974; Krishan 1972; Schroeder 1978). These histological studies also focused on marine invertebrate eggs which showed similar results, producing mutli-nucleate cells (Longo 1972; Copeland 1974; Peaucillier *et al.* 1974; Schroeder 1978). This result also showed the specificity of CB, only denaturing the actin protein in the microfilaments of this contractile ring and no other cellular processes (Longo 1972; Copeland 1974; Peaucillier *et al.* 1974; Schroeder 1978;). Longo (1972) and Peaucillier *et al.* (1974) studied the effects of CB on activated marine invertebrate eggs and



discovered that the release of the polar bodies also used the contractile ring to complete cytokinesis. Hence, CB caused the retention of these bodies. This discovery was of significant importance for ploidy manipulations and thus CB has become a popular chemical for the induction of triploids in both shellfish and fish.

Temperature stress has also been used to induce triploidy. The chemical method is extremely expensive and hazardous, CB is a known carcinogen (Allen *et al.* 1989) as well as inducing higher levels of polyploidy. Temperature inductions were less successful than CB (Beaumont & Fairbrother 1992) but produced acceptable induction results in shellfish like abalone *H. discus hannai* (Arai *et al.* 1986; Fujino 1992), clams (Gosling & Nolan 1989), mussels (Yamamoto & Sugawara 1988; Scarpa *et al.* 1994; Toro & Sastre 1995), oysters (Quillet & Panelay 1986; Yamamoto *et al.* 1988; Wada *et al.* 1989) and scallops (Toro *et al.* 1995).

Temperature inductions of triploidy can be done by either rapidly elevating or lowering the temperatures, between 10 and 20°C, from the normal incubation temperature for the particular species (Beaumont & Fairbrother 1991). It was found that the temperature differential was the important feature of the induction

technique rather than the absolute temperature (Beaumont & Fairbrother 1991).

Hot and cold shocks for inducing triploidy were compared by Arai *et al.* (1986) and Yamamoto & Sugawara (1988), who found that the heat treatment produced better yields of triploid mussels (Yamamoto & Sugawara 1988) but was not significantly better than cold treatment in producing triploid abalone (Arai *et al.* 1986). Temperature shocks are successful at producing triploids as temperature changes denature the microtubules of the polar asters, which separate the chromosomes, and halt some cellular activities (Beaumont & Fairbrother 1991). This has the effect of preventing the release of the polar bodies.

The temperature induction method chosen for *H. midae* was that of heat shock as it appeared to be, generally, a more successful induction method than cold shock..

## **METHODS:**

### **Animals:**

Adult male and female abalone, on the Irvin & Johnson (Pty) (Ltd.) Abalone Farm near Danger Point (34° 40' S 19° 17' E) on the Cape south western coast, were spawned separately. The animals were induced to spawn

using hydrogen peroxide as specified by Hahn (1989). The gametes were then collected and kept separate until they were ready to be treated.

### **CB Induction:**

The eggs were placed into nine sieves (100 $\mu$ m) and divided into three treatment groups consisting of three sieves, viz.: PB1 treatment, PB2 treatment (where polar bodies 1 and 2 were retained respectively) and CON treatment (which was the experimental control). The eggs were then fertilized at a known time and maintained in 17 °C filtered seawater, which was changed every 45 min. for the first 2 hrs post insemination (pi). All zygotes were gently rinsed at 3 min pi to remove as much excess sperm as possible. The PB1 treatment was then treated for 20 min in 0.5 mg.l<sup>-1</sup> CB in seawater (carried in Dimethyl Sulphoxide (DMSO)) starting from 3 min. pi, and rinsed twice (10 min.) in 0.001% DMSO in sea water to remove any remaining CB. Similarly, the PB2 treatment was treated as above for 20 min but starting at 24 min pi. The experiment was then controlled (CON) by treating a group of zygotes in 0.001% DMSO for 20 min at the time of the polar body releases (Fig. 1.1) and also washed twice in the DMSO after treatment. All zygotes were then returned to normal conditions and reared for 120 hrs.

**Polyspermy :**

The possible production of tetraploids from the use of CB was investigated at different concentrations of CB in conditions that either favoured or prevented polyspermy. A comparison of two batches of treatments was set up, which were further subdivided into three treatments with three replicates. The first batch was treated in 0.4 mg.l<sup>-1</sup> and the second in 0.5 mg.l<sup>-1</sup> of CB in seawater. Both of the batches were treated at 3 - 23 min pi in one of the following ways. Either sperm was added with DMSO (control), CB or CB with additional sperm. All added sperm were quantified using a haemocytometer (Mills & McCormick 1992), to keep the concentrations constant (12 X 10<sup>-6</sup> cells ml<sup>-1</sup>). After the induction treatments the zygotes were rinsed in DMSO to remove any excess CB. The larvae were then allowed to develop for 20 hrs before sampling.

**Temperature Induction:**

The eggs were placed in 9 sieves (100 µm) and fertilized at a known time. The sperm was removed after five minutes of incubation. The zygotes were then maintained at 17°C in seawater which was changed every 45 min for the first two hrs pi.

Prior to fertilization, the eggs were separated into 3 groups of 3 sieves. Each group set of three was then treated either at polar body 1 (PB1 treatment) or polar body 2 (PB2 treatment) release or as a control. The zygotes of PB1 treatment were placed in filtered seawater at 30°C, and treated for 10 min., starting from 7 min pi. Similarly, the PB2 treatment zygotes were treated as above but starting from 27 min pi. The control group was maintained at 17°C but moved to another basin of seawater (17°C) for 10 min and then returned. The developing zygotes in all treatments were then returned, after treatment, to fresh filtered seawater at 17°C where they were allowed to develop normally into veliger larvae.

#### **Larval survival:**

Eggs were maintained at 17°C and allowed to hatch. After 20 hrs the larvae from each induction were transferred to separate 75 l larval rearing tanks with a 1 l min<sup>-1</sup> flow rate. The larval numbers were estimated from 5 ml subsamples, taken on the day (20 hrs) they were placed in the tanks. After five days (120 hrs) larval numbers were again estimated, prior to their settlement.

**Ploidy Analysis:**

Samples were taken at 20 hrs pi (approx. 2000 larvae per sample) and 120 hrs pi (approx. 1000 larvae per sample) and transported to the University of Cape Town, and were preserved in Vindelov buffer (Vindelov *et al.* 1983) and frozen at -80°C. The samples were defrosted, providing a single cell suspension, which was centrifuged at 10 000g for ten minutes (Allen 1983) and stained using the Coulter DNA-prep reagents (Coulter PN 6604452). The samples were then analysed on a Coulter Epics Profile flowcytometer (Allen 1983, Johnson *et al.* 1984).

From the resulting histograms (Fig. 3.1 and 3.2) the ploidy induction was estimated using the technique suggested by Downing (1989). The area of the peaks, corresponding to the various ploidy levels, was quantified as the area of 1 mm intervals along the x-axis was measured, integrated and compared to the entire area of the histogram. This provided an estimate of the induction success.

**Statistics:**

All percentage data used were arcsin converted prior to any statistical analysis (Zar 1984). Type I single factor ANOVA's (95% confidence) were

used to analyse CB and temperature induction success and the CB and temperature treated larval survival (Zar 1984). Newman-Keuls analysis (Zar 1984) was then used to differentiate the differences in these tests. Type I two way ANOVA's (95% confidence) analysed the polyspermy and the number of larvae surviving, followed by a Newman-Keuls analysis (Zar 1984).

## **RESULTS:**

### **Flowcytometer histograms:**

Fig. 3.1 shows typical histograms obtained from PB1, PB2 and Control treatments of both the CB and temperature inductions respectively, of the 20 hr samples. These clearly show the different ploidy levels obtained in the differently timed treatments when compared with the control. The control treatment has the characteristic diploid peak (d) with a smaller tetraploid peak (te), indicating rapidly dividing cells in the various phases of the cell cycle (Campbell 1990). In the PB1 and PB2 treatment histograms, the ploidy levels are higher, where the triploid (tr), tetraploid, hexaploid (he) and octoploid (oc) peaks are now visible. In the 120 hr pi samples (Fig. 3.2.) one can still see these

characteristic ploidy levels of the larvae, but there is only one main peak for each of the ploidy levels. This indicated a dormancy of cells.

When comparing the difference in the histograms between the two induction treatments the only difference was at 20 hr pi samples in the PB1 treatments (Fig 3.1), where there was no marked tetraploid peak. Otherwise the histograms all reflect similar trends of triploid inductions, but with varying degrees of success.

#### **CB Induction:**

The results in Table 3.1 shows triploid animals are produced when polar body one (treatment PB1) and polar body two (treatment PB2) are retained. However the PB2 (70.9%) treatment has a significantly greater ( $F=353.6$   $df=F_{1,2,12}$ ) production of triploids than PB1 (48.4%) after 20 hrs pi. It is interesting to note the very high induction rate of tetraploid animals in the PB1 (34.2 %). After five days (120 hrs) the larvae are ready to settle and the second analysis of the induction success indicated significant loss of the tetraploid



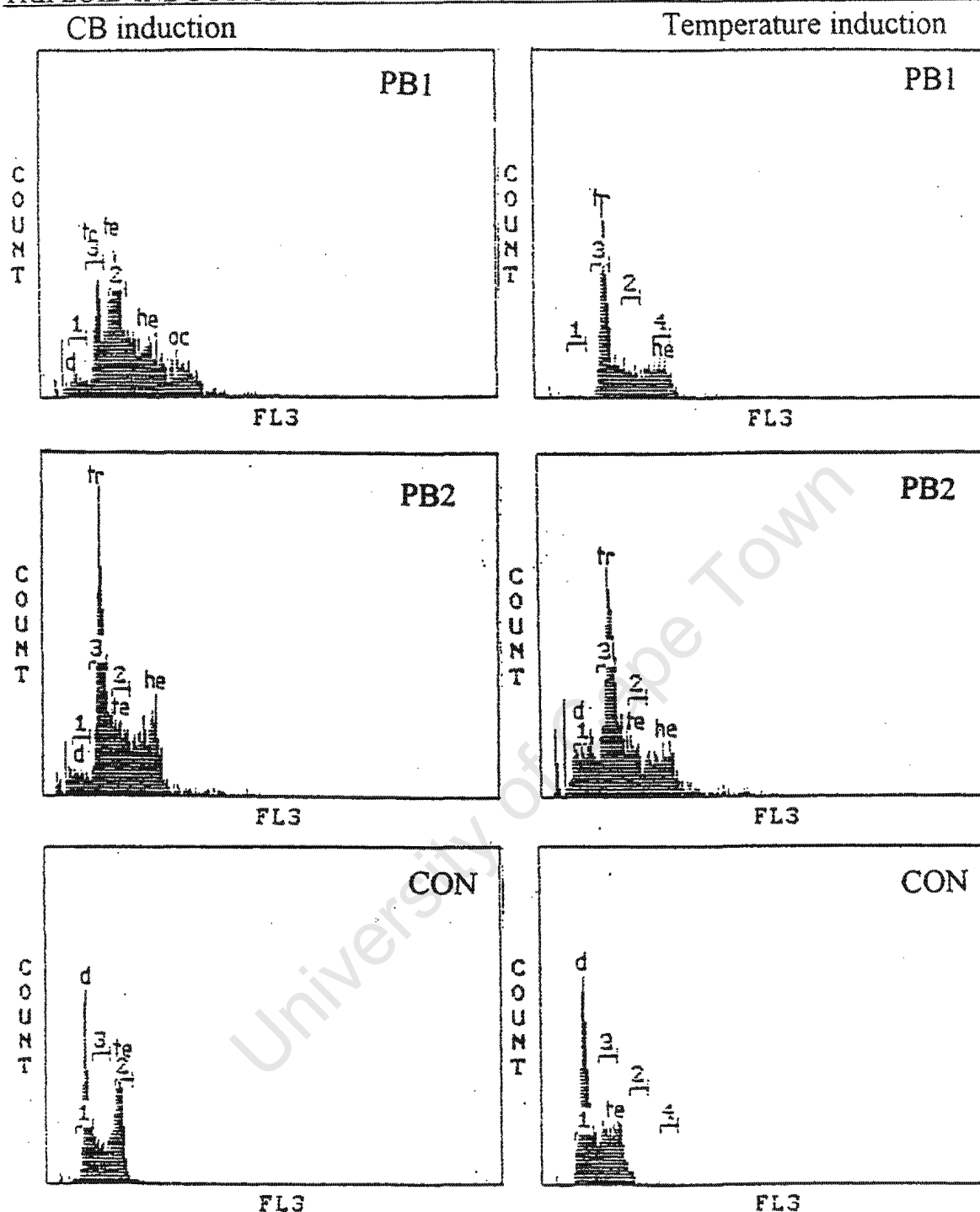


Figure 3.1: Typical histograms produced from the flow cytometric analysis of 20 hrs pi samples from CB and temperature inductions.

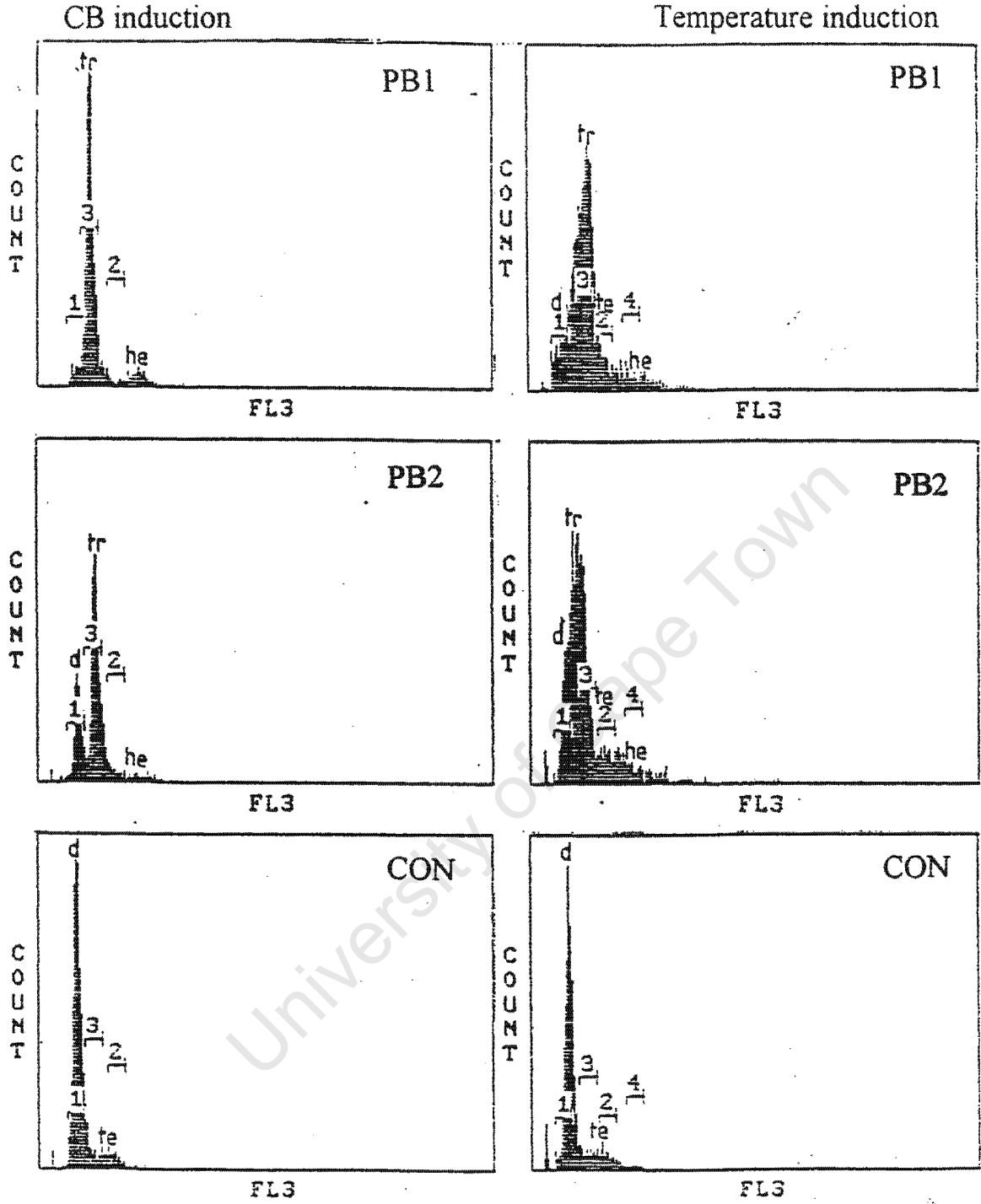


Figure 3.2: Typical histograms produced from the flowcytometry analysis of 120 hrs pi samples from CB and temperature inductions

animals ( $F=353.6$   $df=F_{1,2,12}$ ) in PB1 treatment (4.7%) and PB2 treatment (0%). The percentages of triploids present in PB1 treatment increased (55.5%) but decreased in PB2 treatment (62.4%), indicating the survival of these higher ploidy levels was low.

### Polyspermy and CB concentration:

The results obtained from the polyspermy and concentration experiment are shown in Table 3.2, where the control (Sperm; Table 3.2) showed no change in the ploidy of these zygotes. However the addition of CB changed the ploidy level, by inducing an 86% triploid population at  $0.4 \text{ mg.l}^{-1}$  CB

Table 3.1: The induction success of CB at 20 and 120 hours pi. Where PB1, PB2 and CON refer to the treatments. The ploidy levels are diploid (2n), triploid (3n) and tetraploid (4n).

Treatment	Age (hrs)	% 2n	% 3n	% 4n
CON *	20	100.00		
PB1 *	20	17.35	48.44	34.21
PB2 *	20	26.70	70.89	4.41
CON δ	120	100.00		
PB1 δ	120	39.83	55.46	4.71
PB2 δ	120	37.61	62.39	

\* - significant difference ( $p < 0.05$ ) between 20 hr larvae

δ - significant difference ( $p < 0.05$ ) between 120 hr larvae

Table 3.2: The effects of CB and polyspermy on the percentage polyploid induction. Where Sperm (Control), CB and CB + Sperm refer to the treatments received by a group of zygotes. The ploidy levels are diploid (2n), triploid (3n), tetraploid (4n) and pentaploid (5n).

Treatment	Conc. CB mg.l <sup>-1</sup>	% 2n	% 3n	% 4n	% 5n
Sperm *		100			
CB *	0.4	13.48	86.52		
CB+Sperm *	0.4	5.32	25.41	69.27	
CB *	0.5	18.42	39.00	42.58	
CB+Sperm *	0.5	6.49	15.46	27.95	50.11

\* - significant difference ( $p < 0.05$ ).

in seawater. When excess sperm was present, at this concentration of CB, there was the added presence of tetraploid (69.3%) animals. Changing the concentration of CB to 0.5 mg.l<sup>-1</sup> produced both triploid (39%) and tetraploid (42.6%) animals with just the CB, but when sperm was added a pentaploid (50.1%) population was induced. Treatments all differed significantly from one another ( $F=463.75$   $F=852.85$   $F=114.73$   $df=F_{1,2,12}$ ), indicating that these higher ploidy levels resulted from combined effect of excess sperm and the concentration of CB.

**Temperature Induction:**

Temperature induction was successful where after 20 hrs the PB1 treatment had 93% triploid and PB2 treatment had 86.4% triploid which were significantly different from each other and the control ( $F=106.4$   $df=F_{5,10}$ ) (Table 3.3). After 120 hrs pi the induction remained above 60 % (Table 3.3) in both PB1 and PB2 treatments, but were not significantly different. When comparing these results with the CB induction technique (Table 3.1) one can see that the triploid inductions by CB were significantly less efficient ( $F=18.01$   $df= F_{1,5,35}$ ), especially at the PB1 treatment where only 48.4 % triploidy was induced compared with the

Table 3.3: The induction success of elevated Temperature at 20 and 120 hours pi. Where PB1, PB2 and CON refer to the treatments. The ploidy levels are diploid (2n), triploid (3n) and tetraploid (4n).

Treatment	Age (hrs)	% 2n	% 3n	% 4n
CON *	20	100.00		
PB1 *	20	7.08	92.92	
PB2 *	20	13.58	86.42	
CON δ	120	100.00		
PB1	120	28.91	71.09	
PB2	120	37.53	62.47	

\* - significant difference ( $p < 0.05$ ) between 20 hr larvae

δ - significant difference ( $p < 0.05$ ) between 120 hr larvae

93% in temperature induction. When the 120 hrs pi data were compared (Table 3.1 & 3.3), the PB1 treatments still differed significantly ( $F=18.01$   $df=F_{1,5,35}$ ), however, the PB2 treatments are similar in induction success. It is important to note that the PB1 treatment for temperature induction did not have any higher ploidy levels (such as tetraploids) induced (Table 3.1).

### **Larval survival CB and Temperature:**

Over the five days, the larvae suffered high mortalities (Fig 3.3) where even the control animals only had a 20.1% survival rate. It was noted that there was a difference, although not significant, between CON, PB1 and PB2 treatments, the PB1 treatment having the lowest survival rate (11.6%) followed by PB2 (15.8%).

In the temperature induction, larval survival showed a similar trend to the CB induction, where the PB1 treatment had the lowest survival rate at 6% which was significant different to the PB2 and CON treatments ( $F=11.58$   $df = F_{2,6}$ ), followed by the PB2 treatment at 12% and then the control (CON) larvae having the highest survival rate at 17% (Fig 3.3). These result were compared with the survival of the CB induction technique (Fig 3.3). The larval survival rates appeared somewhat lower. However, when compared to actual larval numbers (

93% in temperature induction. When the 120 hrs pi data were compared (Table 3.1 & 3.3), the PB1 treatments still differed significantly ( $F=18.01$   $df=F_{1,5,35}$ ), however, the PB2 treatments are similar in induction success. It is important to note that the PB1 treatment for temperature induction did not have any higher ploidy levels (such as tetraploids) induced (Table 3.1).

#### **Larval survival CB and Temperature:**

Over the five days, the larvae suffered high mortalities (Fig 3.3) where even the control animals only had a 20.1% survival rate. It was noted that there was a difference, although not significant, between CON, PB1 and PB2 treatments, the PB1 treatment having the lowest survival rate (11.6%) followed by PB2 (15.8%).

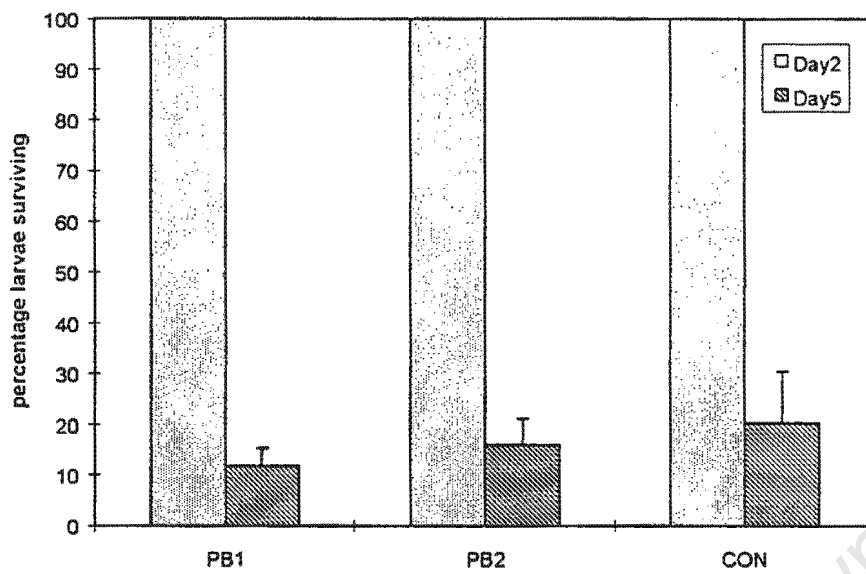
In the temperature induction, larval survival showed a similar trend to the CB induction, where the PB1 treatment had the lowest survival rate at 6% which was significant different to the PB2 and CON treatments ( $F=11.58$   $df = F_{2,6}$ ), followed by the PB2 treatment at 12% and then the control (CON) larvae having the highest survival rate at 17% (Fig 3.3). These result were compared with the survival of the CB induction technique (Fig 3.3). The larval survival rates appeared somewhat lower. However, when compared to actual larval numbers (

Fig 3.4) one can see that the larval numbers did not differ significantly between the two treatments. However it is evident that PB1, PB2 and CON treatments had significantly different larval numbers surviving ( $F=24.9$   $df=F_{1,2;14}$ ), PB1 having the lowest number of larvae (43500). Thus, larval survival appears to be less dependent on the induction method than on which polar body is retained.

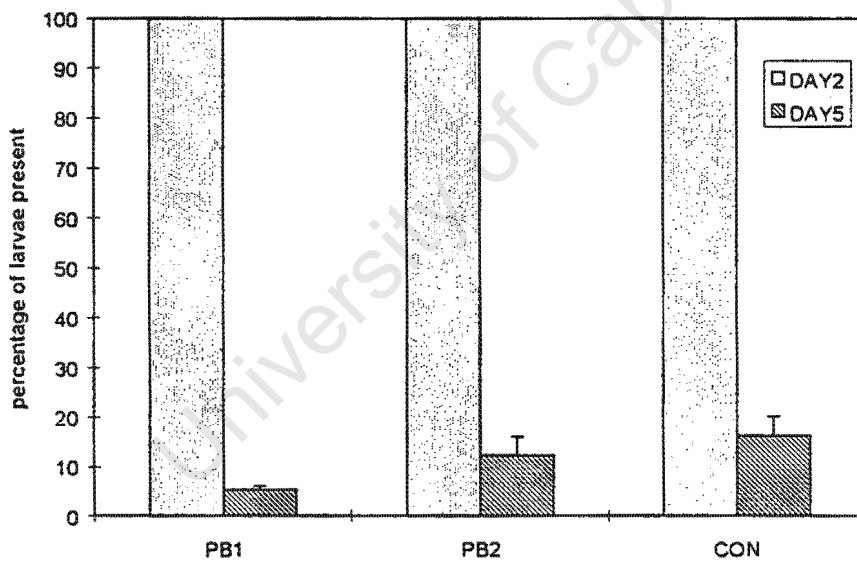
### DISCUSSION:

The induction of triploid *H. midae*, using both CB and elevated temperature shock have been successful, as was the rapid analysis using flow cytometry. The larvae survived through to settlement age with a high degree of triploidy being retained at this time. The results obtained will, therefore, be discussed with reference to their applicability to commercial production of South African abalone.





## CB induction



## Temperature induction

Figure 3.3: The percentage of larvae surviving over 120 hrs for CB and temperature induced triploids, where the error bars are the standard deviation.

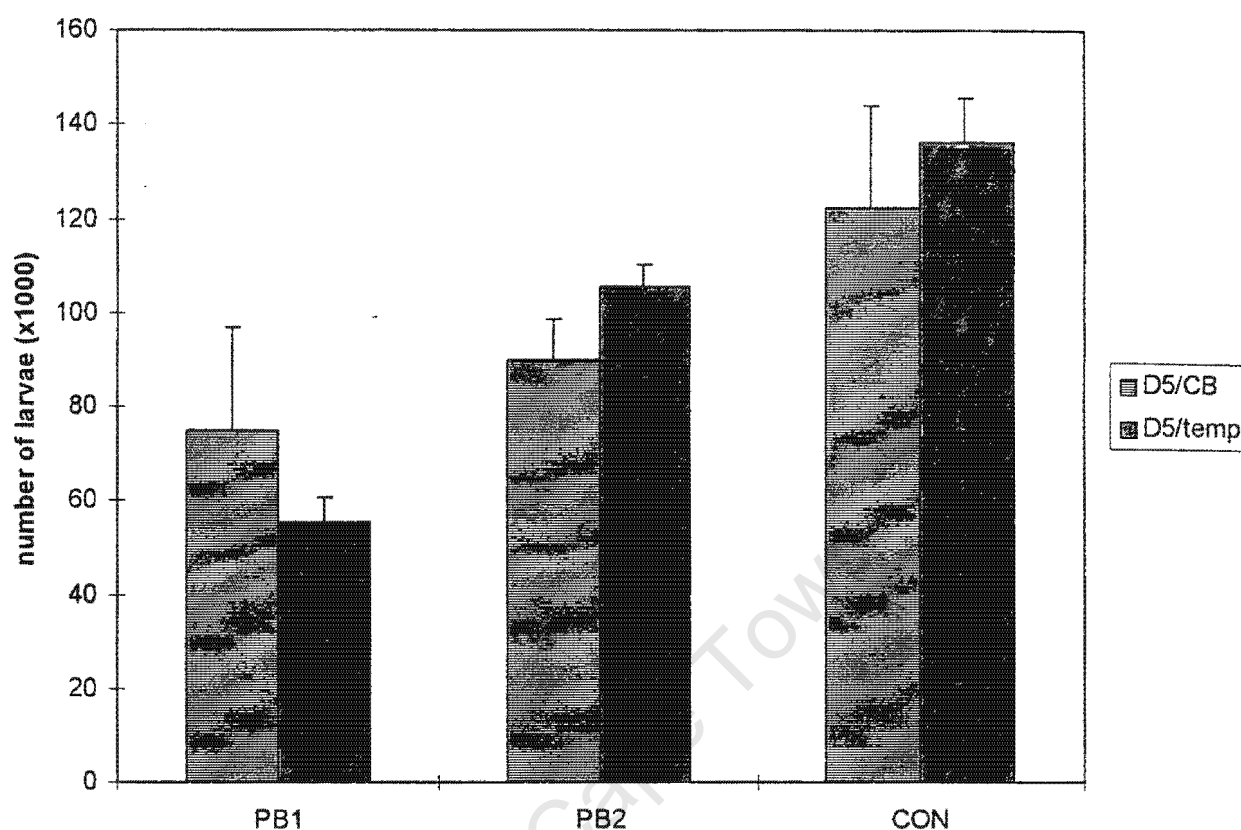


Figure 3.4: Actual numbers of larvae from CB and Temperature induced triploids surviving over the 120 hrs, the error bars represent the standard deviation

The histograms (fig 3.1 & 3.2) reflect typical cell development where a population of cells would be at different phases in the cell cycle (Campbell 1990). The diploid (d) and tetraploid (te) peaks (CON in Fig 3.1) corresponding to the  $G_1$  (period in interphase before DNA replication) and  $G_2$  (period after the DNA replication) phases in the cell cycle (Campbell 1990). These phases are pronounced at the 20 hr pi samples, as the cells are rapidly dividing, and would explain the presence of the hexaploid and octoploid peaks in the PB1 and PB2

treatments. When viewing the 120 hr pi samples the peaks were only pronounced at the G<sub>1</sub> phase, indicating cell dormancy, as the larvae were ready to settle and waiting for a cue (Morse 1984, 1991; McShane 1992). The dormancy of cells at 120 hrs pi would also be useful as it would be a good time to determine the triploid induction success in *H. midae*. Downing's (1989) technique would be most accurate at this time. This would also mean five days of rearing time could be wasted if the treatment was not a success. The 20 hr pi sample would probably be adequate for determining the success of the triploid induction when used commercially.

CB has been found to be a successful agent for the induction of triploidy in *H. midae*, similar to many other groups of shellfish (Stanley *et al.* 1981; Allen *et al.* 1986, Downing & Allen 1987; Beaumont & Contaris 1988; Allen *et al.* 1989; Cooper & Guo 1989). The induction rates obtained for *H. midae* were very comparable with many of the rates found by other researchers as summarized by Beaumont & Fairbrother (1991) which ranged from 50% to 98%. Promising results were seen in PB2 treatment (70.9%) after 20 hrs and after 120 hrs, where both PB1 and PB2 treatments indicated induction rates above 50%. It is also important to note the presence of tetraploids in the PB1 treatment, which was

predicted by a number of researchers using CB as an induction agent (; Stephens & Downing 1989; Grendreau & Grizel 1990; Guo *et al.* 1992, 1994; Allen *et al.* 1994). After five days these tetraploid *H. midae* larvae seem to disappear from the populations, which could be due to starvation of these larvae (Cooper & Guo 1989; Stephens & Downing 1989; Grendreau & Grizel 1990; Guo *et al.* 1992, 1994; Allen *et al.* 1994).

The reasons for the presence of these tetraploids has been suggested by Guo *et al.* (1992) as a characteristic of the eggs, i.e. the pattern in which the chromosomes separate. However it was also suggested that CB affects the membrane and thus allows polyspermy to occur (Longo 1972, 1983; Peaucellier *et al.* 1974). The second explanation seems more applicable in the case of *H. midae*, where experimentation showed how excess sperm in the presence of CB induced higher ploidy levels. This also appeared to be related to the concentration of the CB with which the zygotes were treated, where higher concentrations always produced higher ploidy levels. The effect of CB on the egg's membrane was greater at higher concentrations, allowing any additional sperm to penetrate the eggs and produce tetraploids, even when no excess sperm was added.

Temperature induction has already been carried out successfully on the abalone *H. discus hannai* (Arai *et al.* 1986; Fujino 1992), clams (Gosling & Nolan 1989), mussels (Scarpa *et al.* 1994), oysters (Yamamoto *et al.* 1988) and scallops (Toro *et al.* 1995). The present study indicates that elevated temperatures can also be used to induce triploidy successfully in *H. midae*. This method appeared to be better than CB as it only affected the inner workings of the cell by denaturing the microtubules of the polar asters (Beaumont & Fairbrother 1991); rather than affecting the cell membrane, as with CB (Longo 1972; Copeland 1974; Schroeder 1978). It also avoided the problem of polyspermy. Temperature induction was also considerably cheaper and less hazardous than using CB, giving improved induction rates when properly controlled.

Overall the larval survival rate was low, but this may have resulted from overcrowding in the larval rearing bins. This did not, however, change the result that the larval survival rates in both ploidy treatments were significantly lower than the control. These animals were placed in either highly toxic chemicals (CB (Schroeder 1978) and DMSO (Allen 1989)) or under excessive temperature

stress, which may well have contributed to the low survival rates observed.

Also, these larvae were carrying 1.5 times the normal DNA level and may have been using up their food reserves faster than normal (Hidu *et al.* 1988; Yamamoto *et al.* 1988; Dufy & Diter 1990; Utting & Doyou 1992; Laing & Utting 1994). Another possible explanation for the high mortality rate, particularly in the PB1 animals, may be that they were ready to settle between 6 and 8 hrs earlier, and therefore the five day monitoring period, prior to settlement, would produce an unrealistically high mortality rate.

The survival rate of the triploid larvae was similar in both the CB and temperature treatments, where the lowest survival rate occurred using the PB1 treatment. The actual numbers of larvae surviving (fig 3.4) in both the induction treatments showed no significant difference, suggesting that the carrying capacity of the bins may possibly have been a significant factor in the larval survival. The lower survival rate in both PB1 and PB2 treatments indicated that the stress of the induction treatments, or the excess DNA in the cells, lowered the larval survival rate. Both CB and temperature inductions would produce similar quantities of larvae for settlement.

In conclusion, triploidy can be successfully induced in *H. midae* using either CB or elevated temperatures. CB, however, was extremely toxic and expensive in comparison with elevated seawater temperature. The other disadvantage of CB was that it tended to produce ploidy levels higher than the required triploidy, due to increased polyspermy. Larval survival rates in both CB and temperature inductions were similarly low. These findings indicate that elevated temperatures would be best suited to commercial production of triploid *H. midae*.

On reviewing the ploidy analysis technique, the best time for simple and accurate analysis of induction success would be 120 hrs pi. This may, however, be a concern during commercial production, and the 20 hr pi analysis would provide an adequate indication of the induction efficiency, but will provide an optimistic estimate and should be used with caution.

## **CHAPTER 4:**

### Growth

University of Cape Town



## INTRODUCTION

Triploid research in shellfish was initiated after the success of triploidy on fish farms, producing rapid growth in salmonid fish (Purdom 1983; Chourrout 1984,1987). Research soon followed on valuable shellfish like clams (Allen *et al.* 1982), oysters (Allen & Downing 1986), abalone (Arai *et al.* 1986), mussels (Beaumont & Kelly 1989) and scallops (Tabarini 1984). Triploidy was particularly successful in oysters providing faster growing animals as well as extending the harvests to year round supply (Allen 1988). Further success was achieved in abalone where faster growth of the animals was attained (Arai *et al.* 1986; Fujino 1992; Lerma & McCormick 1995).

Theories on the reason why triploidy results in improved growth, have focused on two aspects of the triploid animals. The first is that sterile animals grow faster because energy that would normally be used for reproduction can be channeled into somatic growth (Allen *et al.* 1986; Davis 1986; Mason *et al.* 1988; Allen & Downing 1990; Hawkins *et al.* 1994). The second theory is based on the concept of heterozygosity and improved growth (Fujio 1982; Hawkins *et al.* 1994; Zouros & Pogson 1994).

Now that it has been shown (Chapter 3) that the induction of triploidy can be carried out successfully, the growth rate of these triploid *H. midae* needs to be compared with that of normal diploid animals. This could then

validate the concept that sterility or heterozygosity (or both) could improve the growth rate of these valuable shellfish.

## **METHODS:**

### **Induction:**

The adult animals were spawned as in Chapter 3 , and triploidy was induced using either CB or temperature. The resulting larvae were reared in 75 l bins for five days.

### **Settlement:**

Separate groups of larvae were placed in 25 l settlement tanks. The tanks and racks were prepared 5 days prior to settlement, and allowed to stand on a 24 hr light cycle to allow diatom growth (Matthews & Cook 1995). Prior to placing the larvae in these bins the excess diatom growth was removed to allow a clean surface for larval settlement on (Matthews & Cook 1995). The larvae were allowed 24 hrs for attachment to the substratum, aeration was started and after 12 hrs water flow was also started at  $0.025 \text{ l min}^{-1}$ .

### **Growth analysis:**

All animals used in the growth analysis were maintained in the farm laboratory on the Irvin & Johnson Abalone farm, with an adequate supply of flowing seawater at 16°C. The abalone were all fed on an excess of fresh kelp (*Ecklonina maxima*) collected from the bay. Two experiments were run. In the first, PB1 and PB2 treatments were compared. At 3 months pi, 50 animals, from each treatment, were placed in separate 5 l jars. Their growth rates were monitored for 5.5 months, by anaesthetizing the animals with MgSO<sub>4</sub> (White 1995) and measuring shell length (sl) at 2 month intervals. In the second experiment, all animals (70) were placed in a 25 l bin and allowed to grow for a year after which shell length was measured every 2 months. All the growth experiments were compared with control diploid animals, which were produced as controls in all induction treatments (Chapter 3).

At the end of the monitoring periods all the animals were anaesthetized and sacrificed, tissue was then removed and preserved in Vindelov buffer and frozen at -80°C, for ploidy determination in the flowcytometer. Tissue biopsies were not used as *H. midae* do not have clotting agents and thus any tissue removed from the animal would result in the animal bleeding to death (Cox 1962; Michelson 1975; Bayne 1983; Hahn 1989; Taylor *et al.* 1994).

**Flow cytometry:**

Tissue samples ( $2 \text{ mm}^3$ ) were defrosted and placed separately in marine phosphate buffered saline (MPBS) and homogenized for 12 sec at 50% power with an Ultra Turax Tissuemiser. The resulting suspension was centrifuged at  $8\,000 \times g$  for 10 min., the supernatant was then decanted and the pellet resuspended in a 0.05% collagenase (Sigma C-0130) in MPBS for 20 minutes at room temperature. The digested suspension was then filtered through a  $100 \mu\text{m}$  mesh filter and neutralized in cold fetal bovine serum (Delta Bioproducts; Product No. 14-501AI). The remaining single cell suspension was again centrifuged ( $10\,000 \times g$ ) for 10 min. The resultant pellet was stained with the Coulter DNA Prep kit (PN 6604452) and diluted to 2 ml sterile citrate buffer.

Histograms resulting from the analysis differentiated between diploid and triploid animals. The results had to be interpolated back, as all the animals were sacrificed at the end of the experiments. The number of triploids present at the final analysis (48 - 50 %) was then used to back calculate the resulting growth curves. This information also allowed for the calculations of daily growth rates ( $\text{mm. day}^{-1}$ ).

**RESULTS:**

Fig 5.1.1 & 5.1.2 present the results from the first experiment, comparing the growth of the PB1 and PB2 treatments. Over 430 days, in both treatments, triploid animals showed an improved growth. However, triploid animals in PB1 treatment appear to have a clearer division of growth curves, between diploid and triploid animals, than in the PB2 treated animals (Fig 5.1.1 & 5.1.2). In the second experiment (Fig 5.2) the same trend was evident. Triploid animals were growing faster than the control (diploid) animals.

These results are only trends but indicated that triploid abalone do grow faster. The growth rates (Table 5.1) shown in all the growth experiments indicate that most of the triploids grew  $0.01 \text{ mm. day}^{-1}$  faster than diploid *H. midae*. Fig 5.1.1 & 5.1.2 and Table 5.1 showed that the PB2 treatment (both diploid and triploid animals) were growing slower than PB1 treatment. The growth rates in the PB2 treated animals, further validated

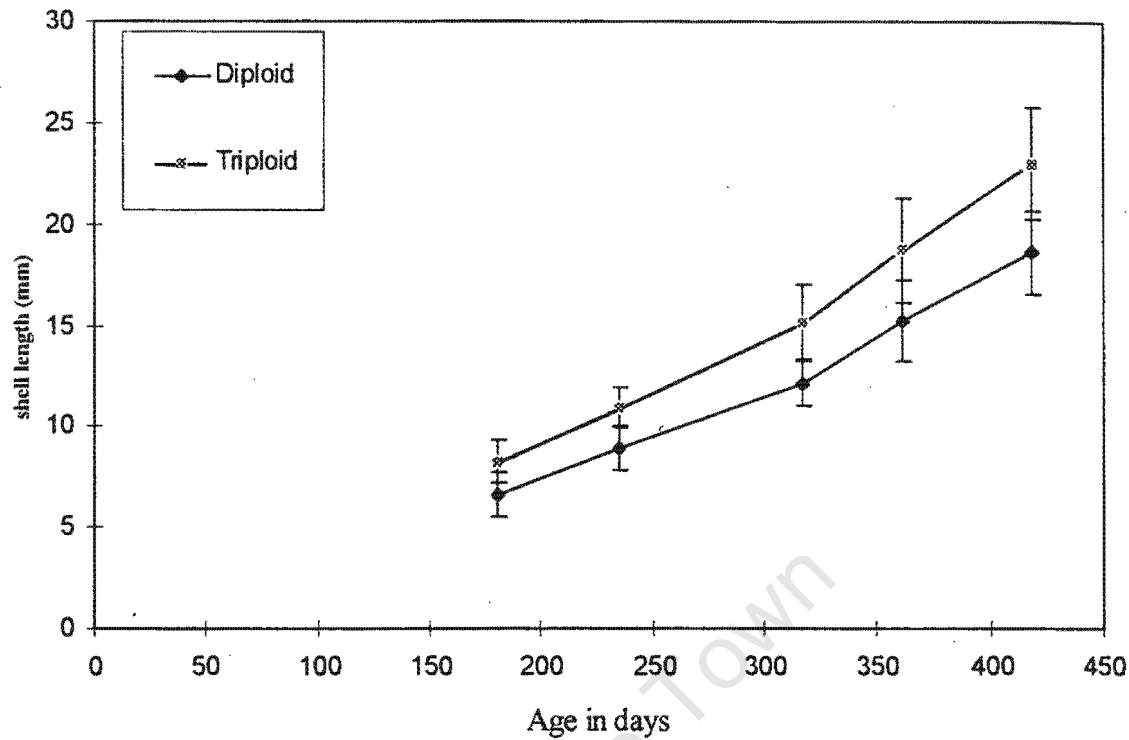


Figure 5.1.1: The growth of the polar body 1 (PB1) retained treatment, where the triploids *H. midae* show a mark growth improvement over the diploid animals.

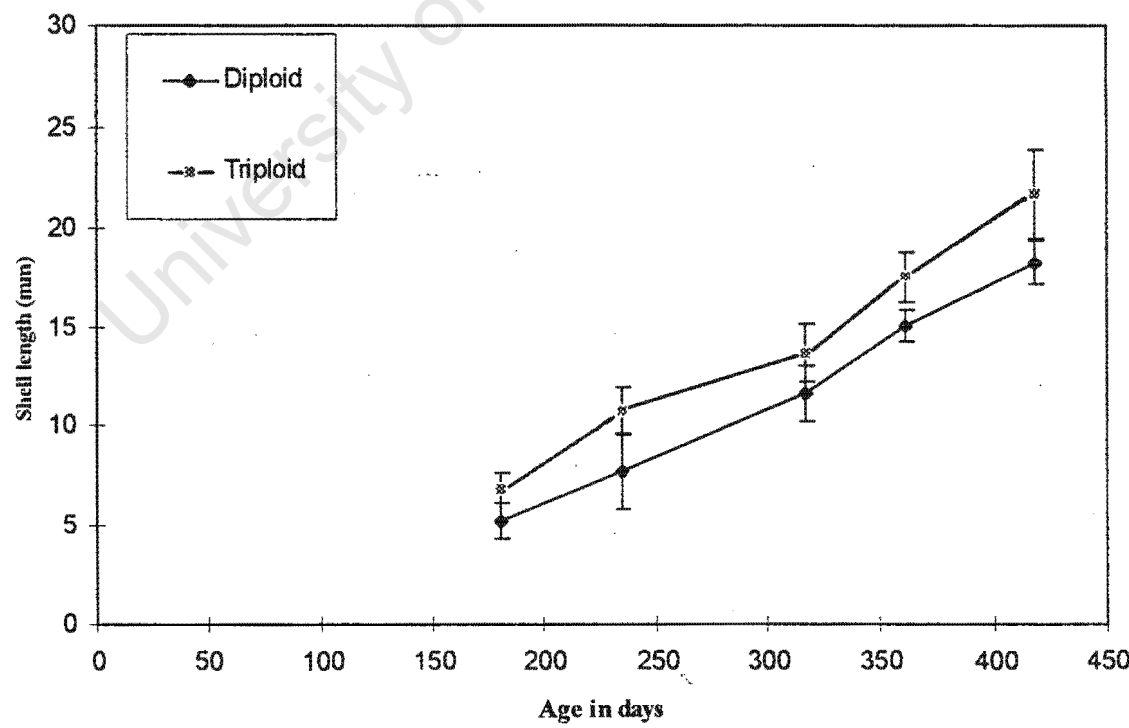


Figure 5.1.2: The growth of the polar body 2 (PB2) retained treatment, where the triploids *H. midae* show a growth improvement over the diploid animals, but not as clearly as in PB1 treatment.

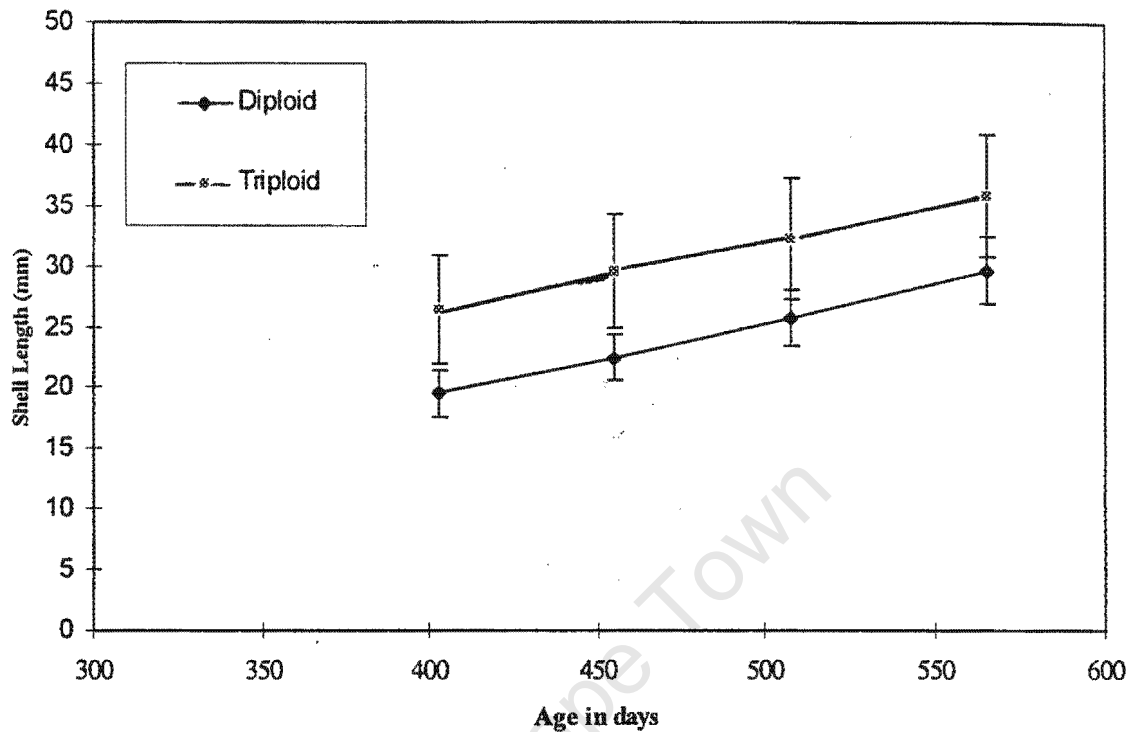


Figure 5.2: The growth of the *H. midae* in the mixed growth experiment, showing that the triploids are growing faster than diploid abalone.

the trends in fig 5.1.2, where the difference in the average growth rates between triploid and diploid animals was not marked, and even the same in some cases. It was interesting to note that the growth rate for PB1 triploids increased to  $0.06 \text{ mm. day}^{-1}$  at the final sample age.

The growth rates in the mixed growth experiment showed an initially higher growth rate, with triploid abalone growing at  $0.02 \text{ mm. day}^{-1}$  faster than the diploid animals, but over the last two sample periods the higher

growth rate of the triploids slowed by  $0.01 \text{ mm. day}^{-1}$ . The overall trend was for the triploid abalone to have a higher growth rate.

Table 5.1: Average growth rates ( $\text{mm. day}^{-1}$ ) of all the growth experiments, where the polar body 1 (PB1) and 2 (PB2) retention treatments, and the mixed growth experiment (M.G.E), show an overall improved growth rate where the *H. midae* were triploid.

AGE (days)	PB1 diploid	PB1 triploid	PB2 diploid	PB2 triploid	M.G.E diploid	M.G.E triploid
181	0.04	0.05	0.03	0.04		
235	0.04	0.05	0.03	0.05		
317	0.04	0.05	0.04	0.04		
361	0.04	0.05	0.04	0.05		
418	0.04	0.06	0.04	0.05		
403					0.05	0.07
455					0.05	0.07
508					0.05	0.06
565					0.05	0.06

## DISCUSSION:

From early research on other valuable shellfish, triploidy has already been shown to produce more rapidly growing animals (Allen 1987; Beaumont & Fairbrother 1991; Chourrout 1987; Purdom 1983; Thorgaard 1983). It has been suggested by a number of researchers, Davis (1986 & 1989), Hidu *et al.* (1988), Hawkins *et al.* (1994) and Mason *et al.* (1988) that these triploidy animals, like abalone (Arai *et al.* 1986; Lerma & McCormick



1995), oysters (Allen & Downing 1986; Mann, Burreson & Allen 1994), and scallops (Komura & Wada 1989) are sterile and thus the energy saved from gonad growth can be channeled into somatic growth. This could explain the faster growth of triploid *H. midae* in both of the above growth experiments. The rapid growth resulting from triploidy would only be expected to become significant after the gonad development, this then does not explain the early (20 mm sl.) manifestation for these faster growth in all cases. It was also noted that the animals in the mixed growth experiment had an interesting slowing of growth rate (Table 5.1). This could be attributed to the measuring methods stressing the abalone (Simpson 1995), hence decreasing the growth rate.

Heterozygosity has been suggested as a reason for increased growth rates (Fujio 1982; Zouros & Pogson 1994), which may explain the early manifestations of the improved growth rate for the triploid *H. midae*. However, other researchers tend to use this explanation to differentiate between the growth rates in PB1 and PB2 treated shellfish (Allen 1987; Beaumont & Fairbrother 1991; Cooper & Guo 1989; Nell et. al. 1995), the former growing faster, especially in *H. discus hannai* (Arai et al. 1986; Fujino 1987, 1992) and oysters (Allen & Downing 1986; Stanley, Hidu & Allen 1984). Polar body 1 triploids are more heterozygous, as they contain

all the maternal DNA, whereas polar body 2 triploids only contain half the maternal DNA. This could explain why the PB1 triploid abalone grew faster ( $0.05 \text{ mm. day}^{-1}$ ) than the PB2 triploids ( $0.04 \text{ mm. day}^{-1}$ ) in the above preliminary investigation. The growth rate of the control abalone were, however slower in the PB2 animals, suggesting possible problems with the housing jars, in which it is possible that flow rates of fresh seawater may have varied between the experimental jars. Thus, further experimentation would be required to verify this result and indicate which of the two triploids, polar body 1 or 2, would be best suited to improving abalone production.

These results provide only preliminary indications of improved growth rates which have not been statistically tested. The trend, however, does indicate improved growth rates in triploid *H. midae* over the diploid animals, which suggest that triploid induction may be a valuable way for improving the slow growth of the South African abalone.

**CHAPTER 5:**

**CONCLUSIONS & RECOMMENDATIONS**

Triploidy has been successfully induced in the South African abalone, using both CB and elevated temperature. Of the two methods, elevated temperature produced the best induction results. The fact that temperature induction is safer and cheaper than using CB, suggest that it may be more appropriate for commercial scale induction (Appendix II). The triploid animals that were produced, survived through the rearing periods, with up to 60% of those that settled being triploid. The growth experiments, although not completely conclusive, suggested that triploid animals appeared to grow faster than the normal diploid animals. The comparison between polar body 1 and 2 triploids was not conclusive as to which would be better for commercial production, but triploids did tend to grow faster than the control.

The use of triploidy in abalone farming in South Africa should be approached cautiously with further investigation into the growth rate of the triploid *H. midae* being necessary. The investigation should be carried out on an actual abalone farm, using temperature as the induction method. The experimental inductions reported here were on a very small scale but could be scaled up to a commercial level using the tank design in Appendix II. The resulting larvae should be reared in much improved rearing bins, with a cone-shaped base, rather than flat bottomed bins, and this could

considerably improve larval survival and their condition up on settlement. Finally, the laboratory conditions must be improved to provide better facilities, such as lighting for diatom growth, and better settlement tanks for experimental work. If these conditions are met then adequate settlement of triploid *H. midae* should be achievable and the suggested improved growth rates of triploid animals could be confirmed.

- Allen S. K. Jr. (1983). Flow cytometry: Assaying experimental polyploid fish and shellfish. Aquaculture **33**: 317 - 328.
- Allen S. K. Jr. (1987). Genetic manipulations - Critical review of methods and performances for shellfish. World Symposium on Selection, Hybridization, and Genetic Engineering in Aquaculture., Berlin. **2**: 127 - 143.
- Allen S. K. Jr. (1988). Triploid oysters ensure year-round supply. Oceanus **31**(3): 58 - 63.
- Allen S. K. Jr. and D. Bushek (1992). Large scale production of triploid oysters, *Crassostrea virginica* (Gmelin), using "stripped" gametes. Aquaculture **103**: 241 - 251.
- Allen S. K. Jr. and S. L. Downing (1986). Performance of triploid Pacific Oysters, *Crassostrea gigas* (Thunberg). 1. Survival, growth, glycogen content and sexual maturation in yearlings. J. Exp. Mar. Biol. Ecol. **102**: 197 - 208.
- Allen S. K. Jr. and S. L. Downing (1990). Performance of triploid Pacific Oysters, *Crassostrea gigas*: gametogenesis. Can. J. Fish. Aquat. Sci. **47**: 1213 - 1222.
- Allen S. K. Jr. and S. L. Downing (1991). Consumers and "experts" alike prefer the taste of sterile triploid over gravid diploid Pacific oysters (*Crassostrea gigas*, Thunberg, 1793). J. Shell. Res. **10**(1): 19 - 22.
- Allen S. K. Jr., S. L. Downing and K. K. Chew (1989). Hatchery Manual for Producing Triploid Oysters. Seattle, University of Washington Press. pp 27.
- Allen S. K. Jr., P. S. Gagnon and H. Hidu (1982). Induced triploidy in soft-shell clam. Cytogenetic and alloenzymic confirmation. J. Hered. **73**: 421 - 428.
- Allen S. K. Jr., S. L. Downing, J. Chaiton and J. H. Beattie (1986). Chemically and pressure induced triploidy in the Pacific oyster *Crassostrea gigas*. Aquaculture **57**: 359 - 360.
- Allen S. K. Jr., H. Hidu and J. G. Stanley (1986). Abnormal gametogenesis and sex ratio in triploid soft-shell clams (*Mya arenaria*). Biol. Bull. **170**: 198 - 210.
- Allen S. K. Jr., M. Shpigel, S. Utting and B. Spencer. (1994). Incidental production of tetraploid Manila clams *Tapes philippinarum* (Adams and Reeve). Aquaculture **128**: 13 - 19.

- Arai K., F. Naito and K. Fujino (1986). Triploidization of the Pacific Abalone with temperature and pressure treatments. Bull. Jap. Soc. Sci. Fish. 52: 417 - 422.
- Barber B. J., R. Mann and S. K. Jr. Allen (1992). Optimization of triploid induction for oyster *Crassostrea virginica* (Gmelin). Aquaculture 106: 21 - 26.
- Baron J., A. Diter and A. Bodoy (1989). Triploid induction in the black scallop (*Chlamys varia* L.) and its effect on larval growth and survival. Aquaculture 77: 103 - 111.
- Bayne C. J. (1983). Molluscan immunobiology. In: The Mollusca. (eds.) A. S. M. Saleuddin and K. M. Wilbur, New York, Academic Press. 407 - 406.
- Beaumont A. R. (1986). Genetic aspects of hatchery rearing of the scallop *Pecten maximus* (L.). Aquaculture 57: 99 - 110.
- Beaumont A. R. and M. H. Contaris (1988). Production of triploid embryos of *Tapes semidecussatus* by the use of Cytochalasin B. Aquaculture 73: 37 - 42.
- Beaumont A. R. and J. E. Fairbrother (1991). Ploidy manipulation in molluscan shellfish: A review. J. Shell. Res. 10: 1 - 18.
- Beaumont A. R. and K. S. Kelly (1989). Production and growth of triploid *Mytilus edulis* larvae. J. Exp. Mar. Biol. Ecol. 132: 69 - 84.
- Britz P. J. (1990). Global Status of Abalone Aquaculture. In: Perlemoen Farming in South Africa. Johannesburg, Mariculture Association of Southern Africa. 20 - 26.
- Buzzi B. and J. J. Manzi (1987). Growth and survival of larval and juvenile polyploid clams, *Mercenaria mercenaria*. J. Shell. Res. 7(1): 151.
- Cadoret J.-P. (1992). Electric field-induced polyploidy in mollusc embryos. Aquaculture 106: 127 - 139.
- Campbell N. A. (1990). Biology. Redwood City, Benjamin/Cummings Publishing Inc. pp. 1165
- Chaiton J. and S. K. Jr. Allen (1985). Early detection of triploidy in larvae of the Pacific Oysters, *Crassostrea gigas*, by flow cytometry. Aquaculture 48: 35 - 43.

- Child A. R. and H. P. Watkins (1994). A simple method to identify triploid molluscan bivalves by the measurement of cell nucleus diameter. Aquaculture 125: 199 - 204.
- Chourrout D. (1984). Pressure-induced retention of the second polar body and suppression of first cleavage in rainbow trout: Production of all-triploid, all-tetraploid, and heterozygous and homozygous diploid gynogenetics. Aquaculture 36: 111.
- Chourrout D. (1987). Genetic manipulations in fish: Review of methods. World Symposium on Selection, Hybridization and Genetic Engineering in Aquaculture., Berlin. 111 - 126.
- Cooper K. and X. Guo (1989). Polyploid pacific oysters produced by inhibiting polar body I and II with cytochalasin B. J. Shell. Res. 8(2): 412.
- Copeland M. (1974). The cellular response to Cytochalasin B: A critical overview. Cytologia 39: 709 - 727.
- Cox K. W. (1962). California abalone, family Haliotidae. Cal. Fish Game Bull. 118: 133.
- Davis J. P. (1986). Energetics of sterile triploid oysters uncouple the reproductive and somatic effort of diploids. J. Shell. Res. 7(1): 114.
- Davis J. P. (1989). Physiology and energetics relating to weight loss and glycogen utilization during starvation in diploid and triploid pacific oysters. J. Shell. Res. 7(3): 549.
- Diter A. and C. Dufy (1990). Polyploidy in the Manila Clam *Ruditapes philippinarum*. II Chemical induction of tetraploid embryos. Aquat. Living Resour. 3: 107 - 112.
- Downing S. L. (1988). Triploid and diploid hybrids between the oysters *Crassostrea gigas* and *C. rivularis* production, detection and potential. J. Shell. Res. 7(1): 156.
- Downing S. L. (1989). Estimating ploidy percentages using oyster larvae: A valuable hatchery management and research tool. J. Shell. Res. 8(1): 320.
- Downing S. L. and S. K. Jr. Allen (1987). Induced triploidy in the Pacific Oyster, *Crassostrea gigas*: Optimal treatments with Cytochalasin B depend on temperature. Aquaculture 61: 1 - 15.



- Duffy C. and A. Diter (1990). Polyploidy in the Manila clam *Ruditapes philippinarum*. I - Chemical induction and larval performances of triploids. Aquat. Living Resour. 3: 55 - 60.
- Fairbrother J. E. (1994). Viable gynogenetic diploid *Mytilis edulis* (L.) larvae produced by ultraviolet light irradiation and cytochalasin B shock. Aquaculture 126: 25 - 34.
- Fallu R. (1991). Abalone Farming. Oxford, Fishing News Books.
- Featherstone S. (1993). Aspects of abalone aquaculture. Conserva. 8: 12 - 14.
- Fujino K. (1987). Japanese aquaculture programme. World Symposium on Selection, Hybridization and Genetic Engineering in Aquaculture., Berlin. 2: 461 - 473.
- Fujino K. (1992). Review of genetics and stock management of Pacific abalone. In: Abalone of the World. (eds.) S. A. Shepherd, M. J. Tegner and S. Guzman del Proi, Oxford, Blackwell Publications. 491 - 503.
- Fujino K., K. Arai, K. Iwadare, T. Yoshida and S. Nakajima (1990). Induction of gynogenetic diploid by inhibiting second meiosis in the Pacific abalone. Nippon Suisan Gakkaishi 56(11): 1755 - 1763.
- Fujio Y. (1982). A correlation of heterozygosity with growth rate in the Pacific oyster, *Crassostrea gigas*. Tokoku J. Agric. Res. 33(2): 66 -75.
- Genade A. B., A. L. Hirst and C. J. Smit (1988). Observations on the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. S. Afr. Jou. Mar. Sci. 6: 3 - 12.
- Gosling E. and A. Nolan (1989). Triploidy induction by thermal shock in the Manila clam, *Tapes semidecussatus*. Aquaculture 78: 223 - 228.
- Grendreau S. and H. Grizel (1990). Induced triploidy and tetraploidy in the European flat oyster, *Ostrea edulis* L. Aquaculture 90: 229 - 238.
- Guo X. and S. K. J. Allen (1994). A unique process for producing tetraploids also demonstrates viability of aneuploid *Crassostrea gigas* Thunberg. (ABSTRACT). J. Shell. Res. 13(1): 278.
- Guo X., W. K. Hershberger, K. Cooper and K. K. Chew (1992). Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the oyster, *Crassostrea gigas*: II. Segregation of chromosomes. Biol. Bull. 183: 387 - 393.

- Guo X., W. K. Hershberger, K. Cooper and K. K. Chew (1993). Artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster *Crassostrea gigas*: I. Induction and survival. Aquaculture 113(3): 201 - 214.
- Guo X., W. K. Hershberger, K. Cooper and K. K. Chew (1994). Tetraploid induction with mitosis I inhibition and cell fusion in the Pacific oyster (*Crassostrea gigas* Thunberg). J. Shell. Res. 13(1): 193 - 198.
- Hahn K. O., Ed. (1989). Hand Book of Culture of Abalone and Other Marine Gastropods. Florida, CRC Press, Inc. pp. 348
- Hamada S. and S. Fujita (1983). DAPI staining improved for quantitative cytofluorometry. Histochemistry 79: 219 - 226.
- Hawkins, A. J. S., A. J. Day, A. Gerard, Y. Naciri, C. Ledu, B. L. Bayne and M. Heral. (1994). A genetic and metabolic basis for faster growth among triploids induced by blocking meiosis 1 but not meiosis 2 in the larviparous European flat oyster, *Ostrea edulis* L. J. Exp. Mar. Biol. Ecol. 184: 21 - 40.
- Hecht T. (1994). Aquaculture in South Africa: Quo Vadis? Newsletter of Aquaculture Association of Southern Africa. May (27) 1 - 3.
- Hecht T. and P. J. Britz (1990). Aquaculture in South Africa: History, Status and Prospects. Pretoria, Aquaculture Association of South Africa. pp 57
- Hidu H., K. M. Mason, S. E. Shumway and S. K. Jr. Allen (1988). Induced triploidy in *Mercenaria mercenaria* L.: Effects on performance in juveniles. J. Shell. Res. 7(1): 202.
- Johnson O. W., P. R. Rabinovich and F. M. Utter (1984). Comparison of the reliability of a Coulter counter with a flow cytometer in determining ploidy levels in Pacific salmon. Aquaculture 43: 99 - 103.
- Komaru A., H. Matsuda, T. Yamakawa and K. T. Wada (1990). Meiosis and fertilization of Japanese pearl oyster eggs at different temperatures observed with fluorescence microscopy. Nippon Suisan Gakkaishi 56: 425 - 430.
- Komaru A., Y. Uchimura, H. Ieyama and K. T. Wada (1988). Detection of induced triploid scallop, *Chlamys nobilis*, by DNA microfluorometry with DAPI staining. Aquaculture 69: 201 - 209.
- Komaru A. and K. T. Wada (1989). Gameteogenesis and growth of induced triploid scallops *Chlamys nobilis*. Nippon Suisan Gakkaishi 55(3): 447 - 452.

- Krishan A. (1972). Cytochalasin-B: Time-lapse cinematographic studies on its effects on cytokinesis. J. Cell. Biol. 54: 647 - 664.
- Laing I. and S. D. Utting (1994). The physiology and biochemistry of diploid and triploid Manila clam (*Tapes philippinarum* Adams & Reeves) larvae and juveniles. J. Exp. Mar. Biol. Ecol. 184: 159 - 169.
- Leighton D. L. (1972). Laboratory observations on the early growth of the abalone *Haliotis sorenseni*, and the effect of temperature on larval development and settling success. Fish. Bull. 70(2): 373 - 379.
- Leighton D. L. (1974). The influence of temperature on larval and juvenile growth in three species of Southern California abalone. Fish. Bull. 72(4): 1137 - 1145.
- Lerma S. and T. B. McCormick (1995). Survival of juvenile triploid abalone. (abstract). Book of abstracts. Aquaculture '95. San Diego. 162.
- Longo F. J. (1972). The effects of cytochalsin B on the events of fertilization in the surf clam, *Spisula solidissima*. J. Exp. Zool. 182: 321 - 344.
- Longo F. J. (1983). Meiotic maturation and fertilization. In: The Mollusca: Development. (eds.) N. H. Verdonk, J. A. M. van den Biggelaar and A. S. Tompa, London, Academic Press Inc. 49 - 89.
- Maguire G. B., N. C. Gardener, J. A. Nell, G. N. Kent and A. S. Kent (1995). Studies on triploid oysters in Australia. 2. Growth, condition index gonad area, and glycogen content of triploid and diploid Pacific oysters, *Crassostrea gigas*, from oysters leases in Tasmania, Australia. (ABSTRACT). Book of Abstracts: Aquaculture '95. San Diego. 355.
- Mann R., E. M. Bureson and S. K. Jr Allen (1994). Growth of triploid *Crassostrea gigas* under natural conditions in the lower Chesapeake Bay. (abstract). J. Shell. Res. 13(1): 279.
- Mason K. M., S. E. Shumway, S. K. Jr. Allen and H. Hidu (1988). Induced triploidy in the soft-shell clam *Mya arenaria*: energetic implications. Mar. Biol. 98: 519 - 528.
- Matthews I. and P. A. Cook (1995). Diatom diet of Abalone post-larvae (*Haliotis midae*) and the effect of pre-grazing over storey. Mar. Freshwater Res. 46: 545 - 548.

- McShane P. E. (1992). Early life history of abalone: a review. In: Abalone of the World. (eds.) S. A. Shepherd, M. J. Tegner and S. Guzman del Proi, Oxford, Blackwell Publications. 120 - 137.
- Michelson E. H. (1975). Cellular defence mechanisms and tissue alterations in gastropod molluscs. In: Invertebrate immunity. (eds.) K. Maramorosch and R. E. Shope, New York, Academic Press. 181 - 195.
- Mills T. S. and T. B. McCormick (1992). Optimum sperm densities for fertilization in three species of abalone (*Haliotis*), Abalone of the World, supplementary papers. Fish. Res. Pap. Dept. Fish. (S. Australia) 24: 42 - 48.
- Morse A. N. C. (1991). How do planktonic larvae know where to settle? American Scientist 79: 155 - 167.
- Morse D. E. (1984). Biochemical and genetic engineering for improved production of abalone and other valuable molluscs. Aquaculture 39: 263 - 282.
- Nell J. A., W. A. O'Conner, R. E. Hand and S. P. MacAdam (1995). Hatchery production of diploid and triploid clams, *Tapes dorsatus* (Lamarck 1818): a potential new species for aquaculture. Aquaculture 130: 389 - 394.
- Newman G. G. (1968). Growth of the South African abalone *Haliotis midae*., Investigational Report (No. 67), Division of Sea Fisheries South Africa. 1 - 60.
- Peaucellier G., P. Guerrier and J. Bergerard. (1974). Effects of Cytochalasin B on meiosis and the development of fertilized and activated eggs of *Sabellaria alveolata* L. (Polychaete Annelid). J. Embryol. Exp. Morph. 31(1): 61 - 74.
- Purdom C. E. (1983). Genetic engineering by the manipulation of chromosomes. Aquaculture 33: 287 - 300.
- Quillet E. and P. J. Panelay (1986). Triploidy induction by thermal shock in the Japanese oyster, *Crassostrea gigas*. Aquaculture 57: 271 - 279.
- Scarpa J., J. E. Toro and K. T. Wada. (1994). Direct comparison of six methods to induce triploidy in bivalves. Aquaculture 119: 119 - 133.
- Scarpa J., D. E. Vaughan and R. Longley (1995). Triploid induction in the American oyster, *Crassostrea virginica*, using 6-DMAP.(Abstract). Book of abstracts. Aquaculture '95. San Diego. 207 - 208.

- Schroeder T. E. (1978). Cytochalasin B, cytokinesis and the contractile ring. In : Cytochalasins - Biochemical and cell biological aspects. Elsevier/North Holland, Biomedical Press. 90 - 112.
- Shen Y.-P., X.-Y. Zhang, H.-P. He and L.-J. Ma (1993). Triploid induction by hydrostatic pressure in the pearl oyster, *Pinctada martensii* Dunker. Aquaculture 110: 221 - 227.
- Simpson B. J. A. (1992). An investigation into which brown algae, *Laminaria pallida* or *Eklonia maxima* is a more suitable diet for the culture of the South African abalone *Haliotis midae*, Honours Thesis, University of Cape Town.
- Simpson B. J. A. (1995). An investigation of diet management strategies for the culture of the South African abalone, *Haliotis midae*, MSc Thesis, University of Cape Town.
- Stanley J. G., S. K. Jr. Allen and H. Hidu (1981). Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin B. Aquaculture 23: 1 - 10.
- Stanley J. G., H. Hidu and S. K. Jr. Allen (1984). Growth of American oysters increased by polyploidy induced by blocking meiosis one but not meiosis two. Aquaculture 37: 147 - 155.
- Stephens L. B. and S. L. Downing (1989). Inhibiting first polar body formation in *Crassostrea gigas* produces tetraploids, not meiotic 1 triploids: J. Shell. Res. 7(3): 550 - 551.
- Tabarini C. L. (1984). Induced triploidy in the bay scallop, *Argopecton irradians*, and its effects on growth and gametogenesis. Aquaculture 42: 151 - 160.
- Taylor J., D. Schiel and H. H. Taylor (1994). The first cut is the deepest: Wounding and bleeding in Black-foot paua (*Haliotis iris*). Seafood New Zealand. 2: 47 - 48.
- Thorgaard G. H. (1983). Chromosome set manipulation and sex control in fish. In: Fish Physiology. London, New York, Academic Press Inc. 405 - 430.
- Thorgaard G. H., P. F. Galbreath and W. P. Young (1995). Evolving perceptions on the utility of chromosome set manipulations in aquaculture.(Abstract). Book of abstracts. Aquaculture '95. San Diego. 226.

- Toro J. E., M. A. Sanhueza, L. Paredes and F. Canello (1995). Induction of triploid embryos by heat shock in the Chilean northern scallop *Argopecten purpuratus* Lamarck, 1819. New. Zeal. J. Mar. Fresh. Res. 29: 101 - 105.
- Toro J. E. and H. D. Sastre (1995). Induced triploidy in the Chilean blue mussel, *Mytilus chilensis* (Hupe 1854), and performance of triploid larvae. J. Shell. Res. 14(1): 161 - 164.
- Uchimura Y., A. Komaru, K. T. Wada, H. Ieyama, M. Yamaki and H. Furata (1989). Detection of induced triploidy at different ages for larvae of Japanese pearl oyster, *Pinctada fucata martensii*, by microfluorometry with DAPI staining. Aquaculture 76: 1 - 9.
- Utting S. D. and A. R. Child (1994). Genetic manipulations of the Manila clam (*Tapes philippinarum*) using cytochalasin B to induce triploidy. Aquaculture 120: 271 - 282.
- Utting S. D. and J. Doyou (1992). The increased utilization of egg lipid reserves following the induction of triploidy in the Manila Clam (*Tapes philippinarum*). Aquaculture 103: 17 - 28.
- Vindelov L. L., I. J. Christensen and N. I. Nissen (1983). Standardization of high-resolution flow cytometry DNA analysis by the use of chicken and trout red blood cells as internal reference standards. Cytometry 3(5): 328 - 333.
- Wada K. T., A. Komaru and Y. Uchimura (1989). Triploid production in the Japanese pearl oyster, *Pinctada fucata martensii*. Aquaculture 76: 11 - 19.
- White H. I. (1995). Anaesthesia in Abalone, *Haliotis midae*, MSc., Rhodes University.
- Yamamoto S., Y. Sugawara, T. Nomura and A. Oshino (1988). Induced triploidy in Pacific oyster *Crassostrea gigas*, and performance of triploid larvae. Nippon Suisan Gakkaishi 39(1): 47 - 59.
- Yamamoto S. and Y. Sugawara (1988). Induced triploidy in the mussel, *Mytilus edulis*, by temperature shock. Aquaculture 72: 21 - 29.
- Zar J. H. (1984). Biostatistical analysis. New Jersey, Prentice-Hall International. pp. 718.
- Zouros E. and G. H. Pogson (1994). Heterozygosity, heterosis and adaptation. In : Genetics and evolution of aquatic organisms. (eds.) A. R. Beaumont, London, Chapman & Hall. 135 - 146.

**APPENDIX I:**

Buffer Solutions Used For Flow cytometry:

**Vindelov Citrate Buffer:**

Sucrose 85.50 g.l<sup>-1</sup> (250 mM)

Tri-sodium citrate.2H<sub>2</sub>O 11.76 g.l<sup>-1</sup> (40 mM)

Dissolved in about 800 ml sterile water

Add 50 ml DMSO

Adjust the pH to 7.2 and make up to 1000 ml

Filter through 0.22 µm filter into a sterile bottle and refrigerate.

**Marine Phosphate Buffered Saline (MPBS):**

Sodium Chloride 11.00 g.l<sup>-1</sup>

Potassium Chloride 0.20 g.l<sup>-1</sup>

Sodium Phosphate (Na<sub>2</sub> HPO<sub>4</sub>. 2H<sub>2</sub>O) 1.15 g.l<sup>-1</sup>

Potassium Phosphate (KH<sub>2</sub>PO<sub>4</sub>) 0.20 g.l<sup>-1</sup>

pH adjusted to 7.2

**Citrate Buffer:**

Sucrose 85.50 g.l<sup>-1</sup> (250 mM)

Tri-sodium citrate.2H<sub>2</sub>O 11.76 g.l<sup>-1</sup> (40 mM)

Dissolved in about 800 ml sterile water

Adjust the pH to 7.2 and make up to 1000 ml

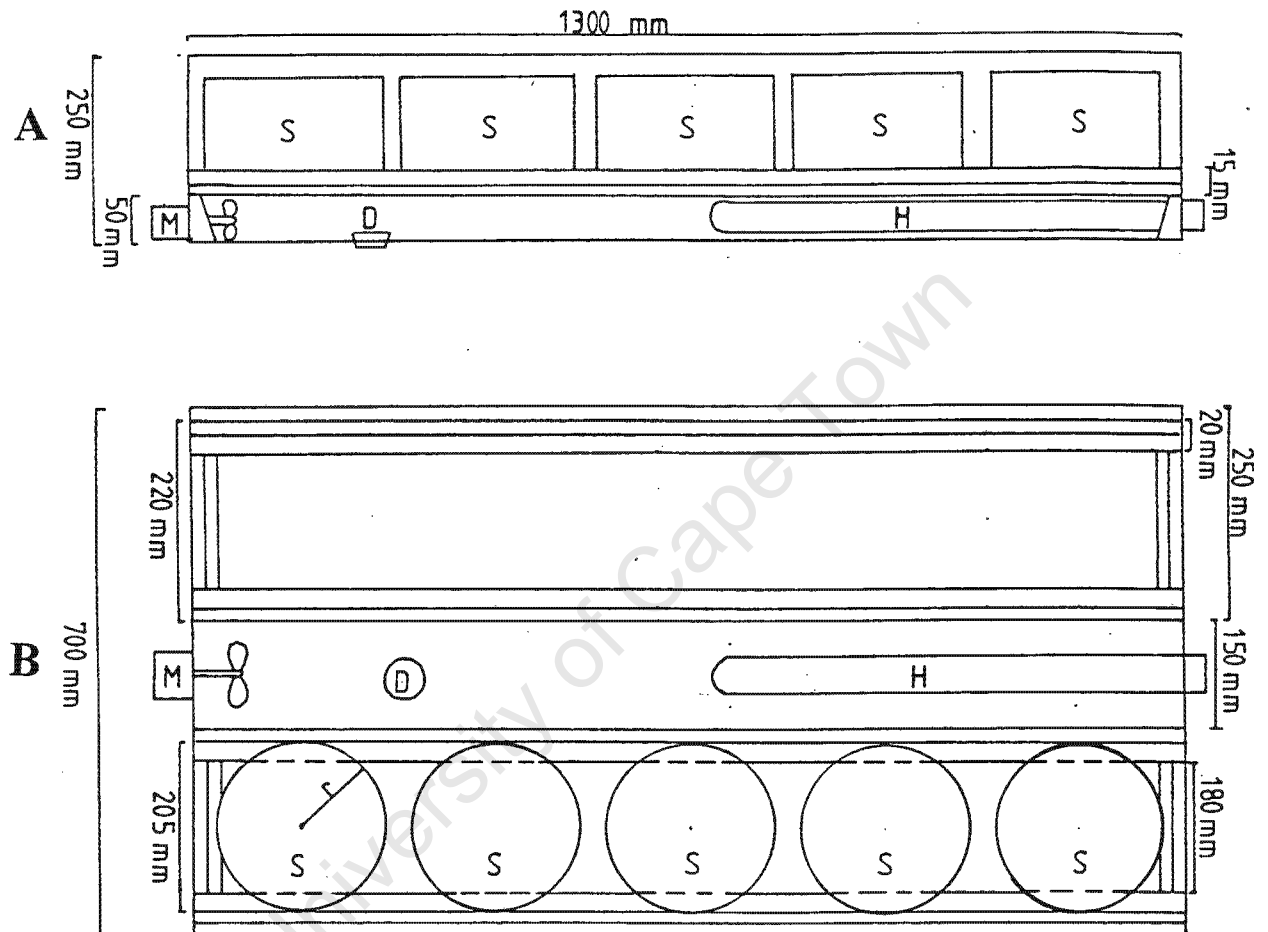
Filter through 0.22  $\mu$ m filter into a sterile bottle and refrigerate.

University of Cape Town



## APPENDIX II:

The following diagram illustrates the design of a tank which could be used for triploid induction of *Haliotis midae* by elevated temperature on a commercial scale. The following diagram presents the possible dimensions of such a tank. A= side view B= plan view.



## Key:

- D Drain for water removal
- H A thermostatically controlled (300 watt) submersible heater
- M Motor to drive a gentle current for homogenous heat distribution
- S Round sieves with a 100 $\mu$ m mesh
- r Radius of sieves (100 mm)